STUDIES OF RAPIDLY INDUCED WOUND ETHYLENE SYNTHESIS BY EXCISED SECTIONS OF ETIOLATED PISUM SATIVUM L., CV. ALASKA

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ABSTRACT

STUDIES OF RAPIDLY INDUCED WOUND
ETHYLENE SYNTHESIS BY EXCISED SECTIONS OF
ETIOLATED PISUM SATIVUM L., CV. ALASKA

By

Mikal Endre Saltveit, Jr.

A rapidly induced, transitory increase in the rate of ethylene synthesis occurred in excised tissue segments from actively growing regions of etiolated barley (Hordeum vulgare L., cv. Maraina), cucumber (Cucumis sativus L., cv. Pioneer), maize (Zea mays L., cv. D-5), oat (Avena sativa L., cv. Victory), pea (Pisum sativum L., cv. Alaska), tomato (Lycopersicon esculentum Mill., cv. Campbell 28), and wheat (Triticum aestivum L., cv. Ionia) seedlings. Cutting the apical region of intact stems or excising 9-mm segments of tissue from near the apex of 7-day-old etiolated 'Alaska' pea seedlings, induced a remarkably consistent pattern of ethylene production. At 25 C, wound-induced ethylene synthesis by 9-mm sections excised 9 mm below the apical hook increased linearly after a 26 min lag, from 2.7 nl g^{-1} hr^{-1} to the first maximum of 11.3 nl g^{-1} hr^{-1} at 56 min. The rate of production then decreased to a minimum at 90 min, increased to a lower second maximum at 131 min, and subsequently declined over a period of about 100 min to about 4.0 nl g^{-1} hr^{-1}.

Removal of endogenous ethylene before the wound response commenced, had no effect on the kinetics of ethylene production. Tissue which had

accumulated large amounts of dissolved ethylene from exposure to high concentrations of ethylene, released it with an exponential decay and no lag period. Therefore, rapidly induced wound ethylene is synthesized by the tissue and is not merely the result of enhanced diffusion of ethylene already present in the tissue through the newly exposed cut surfaces. Previously wounded apical sections did not exhibit a second response when re-wounded. However, these aged sections still possessed an ethylene synthesizing system since treatment with IAA increased ethylene production. No significant correlation was found between wound-induced ethylene synthesis and either CO_2 or ethane production.

Wound-induced ethylene synthesis during the first 90 min was half-saturated at 3.6% O_2, while CO_2 production was half-saturated at 1.06% O_2. Both reactions were saturated at about 10% O_2. Anaerobiosis stopped all ethylene synthesis and delayed the characteristic pattern of wound ethylene synthesis by the length of time freshly excised tissue were deprived of O_2. However, once the rise in wound ethylene synthesis had started, a period of anaerobiosis altered the normal pattern of wound ethylene synthesis when the tissue were returned to air.

Exogenously applied ethylene, or its analogue propylene, stopped wound ethylene synthesis during the time it was applied, but permitted induction of the wound response since wound ethylene synthesis started to rise immediately after the tissue were flushed with air. Lowering the internal concentration of ethylene, by enhanced diffusion at low pressure (130 mm Hg), almost doubled wound ethylene synthesis. Addition of propylene negated the effect of low pressure. Exposure to 4% CO_2 in air reduced wound ethylene production by 30%. It was concluded that the decline in wound ethylene synthesis, which produced the minimum at 90 min,

resulted from negative feedback control of endogenous ethylene on wound ethylene synthesis.

No wound ethylene was produced during the 2 hr after excision at temperatures above 36 C or at 10 C. The low temperature allowed induction of the wound response since tissue immediately produced wound ethylene when warmed to 30 C after 2 hr at 10 C. Temperatures above 36 C stopped wound ethylene synthesis by more drastic means since tissue cooled to 30 C after 1 hr at 40 C required 2 hr before ethylene production returned to normal levels. An Arrhenius plot showed two abrupt changes in the activation energy: one at 15 C and the other at 36 C. The activation energy between these temperatures was 12.1 Kcal $mole^{-1}$ $degree^{-1}$.

The subapical region of etiolated "Alaska' pea seedlings was induced to synthesize wound ethylene by cuts made over 2 cm away, while excision of subapical sections induced wound ethylene synthesis throughout the length of 9- or 16-mm sections. Accumulation of a substance at the cut surface of excised sections, as the result of interrupted translocation, did not initiate or significantly contribute to wound ethylene synthesis. Cutting subapical sections into smaller pieces showed that there are two zones of wound ethylene synthesis. Cells less than 2 mm from the cut surface produced about 30% less wound ethylene than cells greater than 2 mm from the cut surface.

Applying chemicals which are known to perturb IAA transport, or applying IAA at low concentrations had no effect on wound ethylene synthesis. A water-soluble, heat-stable factor diffused from sections in contact with water or agar, which lowered the rate of wound ethylene synthesis, but did not effect the characteristic timing of the wound response. A bioassay was developed to study this diffusible factor. Precursors of

ethylene synthesis in other tissue and in model systems (e.g. methionine, homoserine, homocysteine, propanal) did not prevent the loss of the capacity of subapical sections to produce wound ethylene in the bioassay. A few chemicals maintained normal rates of wound ethylene synthesis in sections in the bioassay (i.e. 10 uM kinetin or benzyladenine, 10 mM Ca, 23 nM triacontanol, and 200 mM sucrose). However, kinetin only was effective if supplied from the beginning of the anaerobic soak in the bioassay; kinetin had no effect if given after the sections had soaked in water for 30 min. Attempts to isolate the factor by solvent partition, dialysis, or molecular sieve or ion exchange chromatography of tissue extracts were unsuccessful.

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By

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Oh, come with old Khayyám, and leave the Wise

To talk; one thing is certain, that Life flies;

One thing is certain, and the Rest is Lies;

The Flower that once has bloomed for ever dies.

Omar Khayyám
XXVI quatrain

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INTRODUCTION

Recent review articles have traced the first observed effect of ethylene on plant growth to 1858 (5,16,101)[1]. However, it was not until 1901 that Neljubov identified ethylene as the active biological agent in illuminating gas. The initial interest in the effects of ethylene on plant growth was soon displaced by investigations into its economically important ability to promote fruit ripening. Although research was difficult because of poor analytical techniques, work continued, albeit slowly, and in the 1930's it was proposed that ethylene was an endogenously produced plant growth substance, similar to a plant hormone (37). However, it was not until after the introduction of gas chromatography as an analytical technique for ethylene in 1959 (23,58) that mounting evidence forced the acceptance of ethylene as a plant hormone.

Despite considerable efforts by a number of researchers to elucidate the site and biosynthetic pathway of ethylene synthesis, and the site and mode of action of ethylene as a plant hormone, these questions remain unresolved. Only in the last few years has research provided support for a proposed in vivo pathway for ethylene biosynthesis from methionine (55,81,82,123). However, the site, terminal steps, and enzymes have yet to be identified.

With refined techniques, ethylene, the only plant hormone yet discovered which is a gas at physiological temperatures, has been shown

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[1] References are listed at the end of "Literature Review".

to affect a wide spectrum of physiological and developmental processes (for a review, see ref. 5 or 16). As with other hormones, plant tissue can modify the activity of endogenous ethylene and thereby modulate its effect on subsequent development. However, since plants do not metabolize or transform an appreciable amount of ethylene into an inactive form (12), control must be exerted through changes in its rate of synthesis and the level of regulator, synergistic, or antagonistic compounds; e.g. auxin, cytokinin, CO_2, or ethylene. Ethylene readily diffuses from tissue, so that a change in its rate of synthesis or diffusivity is quickly reflected in an appropriate change in its endogenous concentration (17, 50). Depending on the stage of development, plant hormones, including ethylene, can either increase or decrease the rate of synthesis or the action of ethylene (45).

Many auxin-induced developmental effects can be attributed to induction of enhanced ethylene synthesis (5). Ethylene in turn, exerts a negative feedback control on the auxin content by regulating auxin distribution and concentration through inhibition of auxin transport and synthesis (18). In climacteric fruit and flowers, ethylene synthesis is controlled until the final phase of development when ethylene appears to exert a positive feedback on ethylene synthesis. The resulting extremely high concentration of ethylene is thought to promote uniform ripening and/or senescence of the tissue. While normal levels of oxygen do not limit ethylene synthesis, ethylene synthesis is extinguished in an anaerobic atmosphere, and its effect is attenuated by elevated endogenous concentrations of CO_2 (21).

Although all plants produce some ethylene, the rate of synthesis varies greatly with regard to the species, the specific tissue, its age,

stage of development, and past and present environment. Stressed plants often have elevated endogenous concentrations of ethylene.

Stress has long been used to intentionally modify the sequence of plant development. Mechanical wounding of immature figs by slashing has been used for the past 5000 years to mimic the natural injury resulting from chalcidoid wasp larva burrowing out of the fruit (46). Bent apple branches have higher internal concentrations of ethylene and come into bearing before non-stressed branches of similar age (77,104). Chemically-induced stress ethylene can assist in harvesting certain crops (e.g. cotton and brussel sprouts) by hastening abscission of leaves (4,33,38,53) or fruit (34).

Many physiological processes of harvested plant commodities are modified by endogenous concentrations of ethylene (14,39). The unavoidable trauma associated with harvesting can enhance ethylene production by some crop plants (15,60,75,90,91,112,117). The resulting elevated levels of ethylene may significantly effect postharvest quality, such as starch to sugar, or sugar to starch conversion, lignin formation, flesh softening, or physiological disorders (14). The increased concentration of ethylene may also hasten the onset of a respiratory climacteric. Increased use of mechanical harvesters will subject fruits and vegetables to more harvest associated injury. A better understanding of the nature of mechanical stress-induced ethylene synthesis could facilitate reduction of injurious effects by the proper postharvest treatment.

This dissertation reports studies on wound-induced ethylene synthesis in excised stem segments from etiolated 'Alaska' pea seedlings. It is hoped that this system will be useful to investigate stress-induced ethylene synthesis.

LITERATURE REVIEW

Induction of Increased Ethylene Synthesis by Stress. An increased rate of ethylene synthesis is elicited in a wide variety of plant tissues subjected to such extrinsic agents as noxious chemicals (2,107), geotropic reorientation (6,104,105), pathogenic microorganisms (41,98), ionizing radiation (1,75), low or freezing temperatures (35,120,124), water imbalance (42,121), and mechanical effects such as abrasion (88), crushing (31,117), flexing (77,104), cutting (46,54,90) and physical restraint of growth (50). Such increases in the rate of ethylene synthesis have been termed wound-, trauma-, stress-, or perturbation-induced ethylene. The term 'stress-induced ethylene' will be used to refer to this general non-ontogenetic change in the rate of ethylene synthesis, while the term 'wound-induced ethylene' will be used to specifically refer to increases in the rate of ethylene synthesis following mechanical injury, such as cutting during excision of tissue.

The rate of stress-induced ethylene synthesis varies with the species, the specific tissue, its stage of development, its past and present environment, and the severity of the stress (for a review, see ref. 5). The rate of synthesis increases with increasing degrees of stress. However, beyond a given level of stress further increases result in decreased synthesis, and if the stress is sufficiently severe, cessation of all physiologically produced ethylene (2,98,121). Ethylene production increased with increasing injury from toxic chemicals (60,119),

pathogenic infections (30,98), and freezing temperatures (43,117). In a similar fashion, cutting tissue into smaller pieces can either increase or decrease ethylene production. Meigh et al. (93) found that cutting apples (Malus domestica Borkh.) or ripe tomatoes (Lycopersicon esculentum Mill.) into 16 equal segments stimulated ethylene production. Cutting apples into 1-cm diameter by 2-mm thick disks reduced ethylene production, while cutting tomato pericarp wall into 4-mm cubes further stimulated ethylene production. Homogenizing either tissue drastically reduced ethylene production. Similar results were reported by Craft (36) and Lieberman and Kunishi (80). Burg and Thimann (25) observed that 4 cm by 1-cm diameter plugs of apple produced ethylene at the same rate as whole apples, but that 1-mm slices produced less-than-half as much ethylene. Respiration was stimulated at each stage of cutting. McGlasson (90) showed that ethylene production was substantially greater from 2-mm than from 4-or 6-mm thick slices of banana fruit (Musa spp., cv. Dwarf Cavendish). Jackson and Osborne (64) reported that cutting 2-cm petiole segments of bean leafs (Phaseolus vulgaris L.,cv. Canadian Wonder) into smaller pieces stimulated ethylene production in proportion to the number of cut surfaces. Imaseki et al. (59) reported that cutting etiolated rice (Oryza sativa L.,cv. Aichi-Asahi) coleoptiles into smaller segments stimulated ethylene production, but that the increase was not proportional to the number of cut surfaces. Increasing the cut surface area of sweet potato roots (Ipomoea batatas L., cv. Norin 1) stimulated ethylene production in proportion to the logarithm of the cut surface area (60).

Placing Coleus blumei Benth. (6) or 'Winesap' apple (104) plants in a horizontal position increased ethylene production. In both species ethylene production reached a maximum 2-to 3-fold increase about 2 days

after reorientation and then slowly returned to normal.

Kinetics of Stress-Induced Ethylene. Stress-induced increases in ethylene synthesis have been reported to occur minutes (54,63,75,90,125), hours (48,56,64,84,91), or days (50,88,105,112) after presentation of the stress. The very rapid increase in ethylene and CO_2 evolution from cut fruits and vegetables may have a trivial explanation. Burg and Thimann (24) showed that within 15 min of sectioning an apple, a 6-fold increase in ethylene and CO_2 evolution occurred. However, if the tissue had been flushed with ethylene-and CO_2-free air, no increase was observed. They concluded that the newly exposed surfaces allowed enhanced gas diffusion from the tissue which initially contained sufficient ethylene and CO_2 to produce the observed increased rate of evolution. Unless this possibility is taken into account and the appropriate controls run, all reports of very rapid increases in the rate of ethylene evolution from cut fruits or vegetables can not be ascribed to increased rates of ethylene synthesis. McGlasson's work with potato tubers and banana fruits provide a good example of this difficulty.

McGlasson (90) showed that cutting potato tubers (Solanum tuberosum L., cv. Bungama) or green banana fruit (Musa spp.) resulted in increased respiration and ethylene production. Ethylene production from 2-mm thick slices of green banana fruit, measured about 1 hr after cutting, was several times higher than from intact fruit. A further increase in ethylene production was detected 4 hr after cutting; reaching a maximum of 2.5 times the initial rate in 6 to 8 hr. Production subsequently declined to the initial wound-induced level in about 1 day, and to barely detectable levels within 2 days. Induced respiration, as measured by CO_2 production, also had two increases; one at 3 to 6 hr and a second at 15 to 20 hr after

cutting. Ethylene production was stimulated 30-fold within 1 hr by slicing potato tubers. There followed a steady and gradual decline during the next 24 hr to a stable rate about 10 times the initial rate. After declining for the first 6 hr, respiration slowly increased to a peak at about 72 hr after cutting. This resembles the pattern of ethylene and CO_2 production reported for sweet potato root slices by Imaseki et al. (60). Since endogenous ethylene was not removed prior to cutting the tissue, it is impossible to say if the initial increases were the result of enhanced diffusion or induced ethylene synthesis. However, the rapid decline in cut potato tubers suggests it was increased diffusion, while the steady rate and subsequent further increase in banana slices suggests it was enhanced synthesis.

Other examples of rapidly induced ethylene evolution include the observation that gashing 16 to 22 day old fig fruits (Ficus sycomorus L., cv. Balami) resulted in a 50-fold increase in the rate of ethylene evolution, which started within 1 hr and remained high for over 6 hr (125). Hanson and Kende (54) showed that wounding 1-day-old rib segments excised from buds of I. tricolor Cav., cv. Heavenly Blue resulted in a 10-fold increase in ethylene production within 1 hr. Lee et al. (75) reported that disks of tomato pericarp produced 20-fold more ethylene 15 to 20 min after cutting than did whole fruit, and that during the next 4 hr ethylene production increased an additional 5-fold.

A similar problem in interpreting the source of increased ethylene evolution is not encountered with small pieces of tissue, e.g. excised sections of stems, roots, petioles, or coleoptiles. The rate of ethylene evolution should closely approximate the rate of synthesis since their large surface-to-volume ratio prevents accumulation of significant concentrations of endogenous ethylene.

Little work has been reported on the kinetics during the first few hr of stress-induced ethylene synthesis from vegetative tissue. Jackson and Osborne (64) reported that ethylene production by segments of petiole or explants from the abscission zone of the primary leaf of 'Canadian Wonder' beans rose steeply 30 min after excision, reached an 18-fold maximum 1.5 to 2.0 hr after excision, and then declined to a stable basal level by about 12 hr. Jackson and Campbell (63) showed that there was a 20 to 30 min delay before the rapid increase in ethylene production from 2-cm petiole segments excised from greenhouse grown 'Moneymaker' tomatoes.

The lag preceding stress-induced ethylene synthesis suggests that activation or synthesis of an ethylene forming system may be required. The lag period preceding auxin-induced ethylene synthesis has been shown, using inhibitors of protein and RNA synthesis, to represent the time required for de novo enzyme synthesis (3,44,57,62,108). Similar experiments with inhibitors suggest that stress-induced ethylene requiring a hr or longer lag period, also requires protein synthesis (2,48).

Sometimes a definite lag period does not precede increased ethylene evolution. Marynick (87) observed that wound ethylene evolution by abscission explants of light-grown 14-day-old cotton seedlings (Gossypium hirsutum L., cv. Acala SJ-1) immediately increased and reached a peak rate of 4.1 ± 2.0 nl g^{-1} hr^{-1} by 1.3 ± 0.5 hr after excision. After 8 to 20 hr ethylene evolution had reached a stable basal rate of 0.2 nl g^{-1} hr^{-1}, which was interrupted by one or two much smaller bursts of ethylene.

Malloch and Osborne (85) reported that excised segments of etiolated oat (Avena sativum L., cv. Maris Quest) and maize (Zea mays L., cv. Golden Bantum) in 10 mM potassium malate buffer at pH 6, produced

an initial surge of wound ethylene which reached a peak 2 hr after cutting. However, the data presented in their paper do not support this statement. Analysis of their data reveals that segments in buffer showed no significant increase in ethylene production during the 20 hr of the experiment. Addition of from 1 to 20 uM IAA to the buffer caused an increase in ethylene production, the timing and degree of which differed among species, tissues (coleoptile or mesocotyl), and concentration of auxin.

Biochemistry of Stress-Induced Ethylene Synthesis. The question of the metabolic source of wound-induced ethylene is not completely resolved. Experiments with inhibitors and feeding labeled compounds suggests that stress-induced ethylene is produced by induction of the same biochemical pathway which uses methionine and produces basal, auxin-induced, and the autocatalytic ethylene of ripening fruit (2,55). However, in the feeding experiments the efficiency of converting methionine into ethylene decreased by 50% in stressed vegetative tissue (2). This indicates that either more unlabeled methionine was being released into the substrate pool, or that a different pathway was induced. The existence of a different pathway is supported by work on the rin tomato mutant and on aging apple disks. Herner and Sink (56) showed that rin tomatoes, although unable to ripen normally and produce ethylene autocatalytically, did produce normal levels of basal ethylene and did respond to being cut with increased CO_2 and ethylene production.

Galliard et al. (48) showed that induction of ethylene synthesis by disks of peel from pre-climacteric apples required O_2 dependent aging and protein synthesis. Ethylene production was stimulated by peroxidation products from linolenic acid, but not by methionine. Meigh et al.

(92) reported an increase in lipoxidase activity in aging apple peel disks which occured just before ethylene production started to increase. Lipid synthesis also increases in aging apple peel disks (47). Fresh disks will produce ethylene if supplied with linolenic acid and lipoxidase (48). Lieberman and Kunishi (78) described a system in which lipoxidase peroxidizes lipase-freed linolenic acid to propanal, which is then converted to ethylene by a copper containing enzyme. However, experiments with C-14 labeled linolenic acid (86) and propanal (9) showed that neither produced labeled ethylene. Lieberman and Kunishi (78) concluded that linolenic acid is not converted to ethylene in living cells. They attributed the stimulated ethylene production from apple peel disks to simple increased peroxidation of linolenic acid.

Translocatable Carbohydrates in Peas. The major translocatable carbohydrate in peas is sucrose (74,99). Quantitative paper chromatographic analysis of an 80% ethanol extract of etiolated germinating pea seeds, showed that the soluble reserve oligosaccharides, stachyose and raffinose, are broken down to glactose and sucrose (74). During the first 100 hr of germination the concentration of stachyose dropped from 93 to 15 mg g^{-1} and raffinose dropped from 31 to 8 mg g^{-1}, while sucrose increased from 77 to 131 mg g^{-1}. Glucose remained constant at 8 to 15 mg g^{-1}, and fructose and galactose were present in trace amounts. It was concluded that galactose actively participated in the metabolic processes of germinating pea seeds, while sucrose was the primary metabolic carbohydrate in the developing axis.

Translocatable Amino Acids in Peas. During germination, the reserve proteins in the cotyledons are hydrolyzed to amino acids which are translocated to and incorporated into the developing axis (11).

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The accumulation of high levels of homoserine in germinating pea seeds has been reported several times (52,71,118). Homoserine is the dominant free amino acid in young seedlings, but declines to low levels 10 to 14 days after germination (71). After 7 days homoserine, which was present at very low levels in ungerminated seeds, accounted for 70% of the free amino acids in green shoots. The predominance of homoserine was not affected by putting developing pea seedlings in the dark; however, other amino acids became detectable (118). Experiments with labeled CO_2, organic acids, or amino acids supplied to cotyledons (11,71), seedlings (52,71), or excised roots (95) resulted in the synthesis of considerable amounts of labeled homoserine, and lesser amounts of asparagine and glutamate. Regardless of the nitrogen source, homoserine, asparagine, and glutamine dominated the amino acids found in the xylem (99). In the axis glutamate was converted to homoserine (11). Homoserine appears to be the major translocatable amino acid in young pea seedlings.

In the cyclic ethylene biosynthesis system proposed by Baur and Yang (10), homoserine and serine are the two necessary inputs needed to maintain ethylene production. Both are converted to methionine, which is then converted to ethylene. However, this may not be the pathway for synthesis of wound ethylene in peas. Burg and Clagett (22) have shown that this pathway only operates in pea sections which have been treated with auxin.

Abscisic Acid in Peas. The concentration of abscisic acid (ABA) increased from around 15 ug kg^{-1} fresh weight in turgid leaves to around 450 ug kg^{-1} in water stressed leaves (94). Wright (121) found that ABA content and ethylene synthesis increased as the water potential of excised wheat seedling leaves increased. Ethylene production paralleled

the rise in ABA to -10.9 bars. Increasing the water potential beyond this resulted in increased ABA, but not increased ethylene production. A 2- to 4-fold increase in ABA in 'Canadian Wonder' bean leaves occurred 20 to 30 min after a water deficit had been induced by blowing warm air over the leaves (122). ABA has been isolated from pea seedlings (61, 103), in which a 20-fold increase occurs when the seedlings lost about 5% of their weight in water (40).

Applied ABA inhibited ethylene production by etiolated wheat leaf sections (83), by normal (79) or IAA-treated etiolated mung bean sections (70), and by etiolated 'Alaska' pea stem sections (49). It stimulated ethylene production by etiolated mung bean sections (70), by cycloheximide treated flavedo of citrus fruits (32), by senescent leaf disks (79), and by pulvinar explants of bean (65).

Auxin in Peas. Indole-3-acetic acid (IAA) is transported basipetally by an auxin specific system (97) from its site of synthesis in the apical region (111) at a velocity of between 5 to 15 mm hr^{-1} for intact plants or excised segments (51,89,96,110). Ethylene does not inhibit basipetal auxin transport in subapical sections of etiolated 'Alaska' pea seedlings unless the plants have been pretreated with ethylene for a few hr (5,19). Since inhibition only slightly reduced the velocity of auxin transport, it is thought to work through increased auxin immobilization (13,19). Scott and Briggs (109,110,111) were able to obtain diffusible auxin from excised segments of light-, but not dark-grown pea stems. They attributed this failure to auxin inactivation at the cut surface caused by high levels of IAA oxidase in the etiolated pea seedlings. Stem tissue has been shown to convert endogenous IAA to indole-3-acetyl-aspartic acid (IAAsp)(96). Short-term ether extractions

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were used to isolate the free endogenous IAA from light- and dark-grown pea stems. They showed that the apical 10 cm of light-grown stems contained much more free auxin, distributed with only a slight basipetal decrease, than does etiolated stems in which a rapid basipetal decrease occurs. Removal of the apical bud from light- or dark-grown pea seedlings results in a progressive basipetal reduction in free auxin which completely drains the stem in 12 to 18 hr. Application of IAA to the cut apex results in a progressive basipetal increase of free auxin to near normal levels.

Auxin, when applied above a threshold concentration, stimulates ethylene production in almost all plant tissue examined (5). Abeles and Rubinstein (7) were the first to show that exogenous auxin (50 uM NAA) increased ethylene synthesis by stem sections of etiolated 'Alaska' pea. Later investigators (see ref. 5) confirmed this observation and further showed that stimulated ethylene production is a function of the concentration of IAA applied to the root or stem of etiolated peas. Concentrations of IAA above 1 uM for subapical stem sections, or 0.01 uM for root sections stimulated ethylene production within 15 to 30 min for roots (28) and within 1 hr for stem sections (18). A 6 hr preincubation of hypocotyl sections from etiolated mung bean seedlings in 10 uM IAA caused an increase in ethylene production, and eliminated the 1 hr lag in stimulating ethylene production when they, as compared to similar sections preincubated in IAA-free solution, were transfered to 500 uM auxin (108). Abeles (3) tested the hypothesis that auxin-induced ethylene production is the result of de novo protein or RNA synthesis by exposing segments of etiolated 'Red Kidney' beans, 'Russian Mammoth' sunflower (Helianthus annuus L.), or 'Burpee's Barbecue Hybrid' corn

(Zea mays L.) to auxin plus or minus inhibitors of protein or RNA synthesis. While none of the four inhibitors (actinomycin D, puromycin, 2-thiouracil, and p-fluorophenylalanine) had an effect on endogenous rates of ethylene synthesis, actinomycin D reduced stimulated ethylene production by 84%; back to the endogenous level. Other researchers have confirmed the fact that IAA-induced ethylene production requires protein synthesis (45,68,108,115). In etiolated subapical sections of 'Alaska' pea, IAA-induced ethylene synthesis requires the formation of short lived RNA which is required for the synthesis of highly labile protein which controls the rate of ethylene synthesis (57,68).

Ethylene production by vegetative tissue, whether endogenous or stimulated by applied auxin, is proportional to the concentration of free auxin in the tissue (7,20,29,68). The concentration of free endogenous IAA is directly dependent on the rate of synthesis and/or uptake, and inversely dependent on the rate of conjugation and decarboxylation (68,72). Destruction of applied IAA takes place by decarboxylation at the cut surface before it can enter the tissue and be conjugated to indole-3-acetyl-aspartic acid (IAAsp)(97) which is inactive in inducing ethylene production (68).

Kinetin slightly increased ethylene production from etiolated mung bean hypocotyl sections, but a remarkable synergistic effect occurred on IAA-induced ethylene production (45). Lau and Yang (72) showed that this resulted from enhanced uptake and decreased IAA conjugation to IAAsp.

Cytokinins in Peas. Extracts with cytokinin activity have been isolated from pea roots (114), root nodules (116), young pea seedlings (8, 126), developing pea seeds (26), xylem exudate from the field pea (2), and the blanching water used in canning peas (106). Short and

Torrey (114) identified the major active factor in pea root extracts as zeatin, or a closely related compound, by the use of paper chromatography before and after chemical modification. The chromatographic region corresponding to IPA had much less activity. Shibaoka and Thimann (113) were unable to isolate an active cytokinin fraction from pea root tips because their extract contained a ninhydrin positive inhibitor of their bioassay. Of 22 amino acids tested, at concentrations of 30 mM with 1.4 uM kinetin, only L-serine (at 26%), L-cysteine (at 37%), and L-alanine (at 45%) gave inhibitions greated than 50% of the kinetin control. L-arginine strongly antagonized L-serine promoted proteolysis, while having no activity itself. The effect of homoserine was not investigated.

Cytokinin activity has been found in acid-hydrolyzed pea transfer RNA (8,114). Babcock and Morris (8) identified cis-zeatin by mass spectroscopy and IPA by gas chromatography from hydrolyzed pea t-RNA. Natural cytokinin-active ribonucleosides have been found in t-RNA from cytokinin-dependent tobacco callus cultures supplied with only synthetic cytokinin (76). Although natural cytokinins are found in specific t-RNA's (serine t-RNA and tyrosine t-RNA), biological activity of synthetic unincorporable cytokinins argues against the idea that the mechanism of action depends on incorporation into t-RNA (100).

A slight increase in ethylene production resulted from the application of either 10 uM kinetin or 10 mM Ca^{++} to etiolated hypocotyl sections of mung bean, but a remarkable synergistic increase occurred when they were applied together (73). No synergistic effect was observed with application of kinetin and GA_3.

Gibberellins in Peas. Radley (102) showed that ethanol extractable gibberellins are uniformly distributed throughout the shoot of Pisum sativum L. seedlings. The levels of gibberellin-like activity have been shown to be the same in light- and dark-grown pea plants (67, 69). Both A_1- and A_5-like gibberellins were obtained by methanol extraction of pea seedlings, however, only A_1-like activity was found by diffusion extraction into 1.5% (w/v) agar (67). This was explained by the fact that GA_5 may be a non-mobile precursor to GA_1 in pea plants. Jones (66) reported an aqueous extraction of pea seedlings which confirmed the presence of A_1- and A_5-like compounds, and also extracted two more gibberellin-like substances.

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SECTION ONE

STUDIES OF RAPIDLY INDUCED WOUND ETHYLENE SYNTHESIS BY EXCISED SECTIONS OF ETIOLATED *PISUM SATIVUM* L., CV. ALASKA

I. CHARACTERIZATION OF THE WOUND RESPONSE

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ABSTRACT

A rapidly induced, transitory increase in the rate of ethylene synthesis occurred in excised tissue segments from actively growing regions of etiolated barley (Hordeum vulgare L., cv. Maraina), cucumber (Cucumis sativus L., cv. Pioneer), maize (Zea mays L., cv. D-5), oat (Avena sativa L., cv. Victory), pea (Pisum sativum L., cv. Alaska), tomato (Lycopersicon esculentum Mill., cv. Campbell 28), and wheat (Triticum aestivum L., cv. Ionia) seedlings. Cutting the apical region of intact stems or excising 9-mm segments of tissue from near the apex of 7-day-old etiolated 'Alaska' pea seedlings, induced a remarkably consistent pattern of ethylene production. At 25 C, wound-induced ethylene synthesis by 9-mm stem sections excised 9 mm below the top of the apical hook increased linearly after a 26 min lag, from 2.7 nl g^{-1} hr^{-1} to the first maximum of 11.3 nl g^{-1} hr^{-1} at 56 min. The rate of production then decreased to a minimum at 90 min, increased to a lower second maximum at 131 min, and subsequently declined over a period of about 100 min to about 4.0 nl g^{-1} hr^{-1}.

Removal of endogenous ethylene before the wound response commenced, had no effect on the kinetics of wound ethylene production. Tissue which had accumulated large amounts of dissolved ethylene from exposure to high concentrations of ethylene, released it with an exponential decay and no

lag period. Therefore, rapidly induced wound ethylene is synthesized by the tissue and is not merely the result of enhanced diffusion of ethylene already present in the tissue through the newly exposed cut surfaces. Previously wounded apical sections did not exhibit a second wound response when re-wounded. However, these aged sections still possessed an ethylene synthesizing system since treatment with IAA increased ethylene production. No significant correlation was found between wound-induced ethylene synthesis and either CO_2 or ethane production.

INTRODUCTION

Ethylene production increases in a wide variety of plant tissues subjected to stress induced by noxious chemicals (2), pathogenic organisms (19), ionizing radiation (1), water imbalance (23), or mechanical injury (16). Abeles (3) has reviewed stress ethylene production. Stress-induced increases in ethylene production occur minutes (8,10,12, 17,24), hours (6,9,11,14,18), or days (7,16,21,22) after presentation of the stress. Appropriate controls are necessary to ascertain whether rapid increases in the rate of ethylene evolution result from facilitated diffusion of endogenous ethylene through the newly exposed cut surfaces, or from induced synthesis. Burg and Thimann (5) showed that in sliced apples the initial surge in ethylene and carbon dioxide evolution was the result of endogenous levels of these gases. Increased evolution of ethylene after a definite lag period suggests induced synthesis. This is supported by studies with inhibitors which showed that protein synthesis is required during the long lag-period preceeding stress ethylene synthesis (2,6).

Little work has been reported on the kinetics of rapidly induced ethylene production from stressed tissue (10,11). This paper presents a detailed kinetic study of ethylene production from mechanically injured tissue during the first 4 hr after excision.

MATERIAL AND METHODS

Preparation of Plant Material. Seeds of Pisum sativum L., cv. Alaska were imbibed in aerated tap water for 6 hr at 23 $\pm$ 2 C and

planted in moist vermiculite. All subsequent manipulations were performed in the dark or under dim green light. The growth cabinet was maintained at 24 $\pm$ 1 C and continuously flushed with humidified ethylene-free (<1 nl l^{-1}) air. Seven-day-old etiolated seedlings, with 3 to 8 cm long third internodes and with reflexed apical hooks were selected. Apical stem segments were cut 9 mm below the top of the apical hook and included the apical meristem, plumule, and apical hook. Subapical stem segments were excised from the region 9 to 18 mm below the apical hook and consisted primarily of elongating cells. Internodal stem segments were excised from the second internode and consisted primarily of mature differentiated cells. Root apical segments were cut 9 mm from the root apex and included the root cap, apical meristem, and adjacent regions. Except as noted, all experiments used freshly excised 9-mm tissue segments. Some experiments used stem segments which had dissipated their initial wound response (aged segments). Tissue from other parts of the plant were used and are described in the appropriate text.

Other plant materials were used to investigate the ubiquitiousness of the wound-induced phenomenon. These included 9-mm sections excised from the region 9 to 18 mm from the shoot apex of red bud (Cercis canadensis L.), honeysuckle (Lonicera flexuosa var Halliana Dipp.), and Forsythia viridissima Lindl. growing under natural conditions. Tissue from etiolated plants prepared in the same manner as the peas were also used. They included excised cotyledons and hypocotyl segments from seven-day-old cucumber seedlings (Cucumis sativus L., cv. Pioneer), apical stem segments from seven-day-old tomato seedlings (Lycopersicon esculentum Mill., cv. Campbell 28), and apical coleoptile segments from five-day-old seedlings of oats (Avena sativa L., cv. Victory), barley

(Hordeum vulgare L., cv. Maraini), winter wheat (Triticum aestivum L., cv. Ionia), and corn (Zea mays L., cv. D-5).

Experimental Procedure. The kinetics of wound-induced ethylene synthesis were studied using a flow-through system and a static system in which evolved ethylene accumulated. The flow-through system used tissue segments enclosed in opaque glass tubes which ranged from 0.8- to 26-ml, depending on the experiment, and which were capped with serum stoppers. Humidified ethylene-free air (<1 nl l^{-1}), or other gases, were admitted at a positive pressure of 2 cm Hg through a hypodermic needle inserted in the bottom serum stopper. Samples were taken through the top serum stopper at regular intervals with gas-tight syringes to give flow rates of either 1 or 2 ml min^{-1} and immediately injected into a gas chromatograph which used nitrogen at 60 C as the carrier gas, was equipped with a 2 mm by 1 m column of activated alumina, and a flame ionization detector. The gas chromatograph readily detected ethylene at concentrations of 1 nl l^{-1} in a 1 ml sample with a retention time of ca. 25 sec.

Carbon dioxide and oxygen were determined by injecting samples into a gas chromatograph which used helium at 70 C as the carrier gas, was equipped with a 6 mm by 0.6 m silica gel and a 6 mm by 3 m molecular sieve column mounted in parallel, and a differential thermal conductivity detector. Ethylene, carbon dioxide, and oxygen concentrations were determined by comparing peak heights with those produced by a standard gas mixture. Ethane production was analyzed in some experiments and in the absence of an ethane standard, was quantitatively related to ethylene by comparing their relative peak heights within an experiment.

In the first series of experiments, 3 sets of 15 excised 13 cm

etiolated pea stems were held for 140 min in 1.5 cm by 15 cm opaque glass tubes which contained 2 ml of water and were continuously flushed with humidified ethylene-free air. The stems were then wounded with 5 cuts half-way through the stem, perpendicular to the stem axis, and between 9 and 18 mm below the apical hook. Ethylene evolution was followed for 70 min after wounding by taking 2 ml samples every 2 min. This experiment was repeated 3 times.

This system proved inadequate to accurately characterize the kinetics of wound ethylene synthesis. Limited gas exchange, resulting from the large ratio of sample chamber volume (26 ml) to sample volume (2 ml), made detection of rapid changes in the rate of synthesis, as well as calculation of the absolute rate of synthesis very difficult. Smaller sample chambers were therefore used to increase the systems responsiveness.

Except as noted, all subsequent experiments were conducted with 9-mm segments of tissue excised from the region 9 to 18 mm below the top of the apical hook and enclosed in 0.8- to 2.2-ml glass sample containers. The exchangeable gas volume was further reduced by the approximately 0.4 ml volume of the tissue. Decay of ethylene from a spiked system showed that under actual conditions, approximately 90% of the gas was removed by every 2 ml sample. Smaller tissue samples also permitted study of specific regions of a plant and maintenance of an adequate flow rate by repetitive sampling. The latter facilitated rapid gas exchange, yet allowed sufficient ethylene to accumulate for analysis. Three treatments were run simultaneously with a sampling rate of 2 ml $(2\ min)^{-1}$ by injecting the samples into the gas chromatograph on a staggered 40-sec schedule.

Analysis of Data. Whenever possible, actual data from a representative experiment was used to study the kinetics of wound-induced ethylene synthesis. However, when there was a large amount of variability, curves were either fitted manually or drawn using data subjected to a 5-point curve-smoothing equation. The first peak of ethylene production was reasonably symmetrical. A second degree polynomial was fitted to data from the first peak by the method of least squares. The quadratic equation was then used to calculate the time of maximum ethylene evolution (the first derivative) and the amount of ethylene evolved during a standard interval about the maximum (the area under the curve as calculated from the integral equation, and bounded by the time of maximum ethylene production plus or minus 30 min). These parameters and similar data from accumulation studies were subjected to analysis of variance. Each experiment reported was repeated at least twice.

RESULTS AND DISCUSSION

Preliminary Observations. Ethylene production by 13-cm etiolated pea stems declined slightly after the apical region was cut, increased after about 25 min, and became significantly greater than the noninjured control after a lag-period of about 30 min (Fig. 1). Production by the noninjured control remained virtually constant during the experiment. Similar patterns were observed in three experiments. Two-hr accumulation experiments showed that 3 cuts perpendicular to the stem axis and half-way through the stem were as effective in eliciting wound ethylene (5.3 nl g^{-1} hr^{-1}) as were 3 cuts parallel to the stem axis (5.5 nl g^{-1} hr^{-1})

Ethylene accumulation experiments were done with tissue enclosed in 25-ml Erlenmeyer flasks, 21-ml test tubes, or 3- or 5-ml plastic gas-tight syringes. Internal humidity was maintained near saturation with moist filter paper, and carbon dioxide was absorbed by strips of filter paper moistened with a freshly prepared saturated solution of KOH.

The presence of the ethylene synthesizing system was studied using aged and freshly excised subapical stem segments. The tissue was treated with 2 ml of water or 10 uM IAA in 25-ml Erlenmeyer flasks. Gas samples were taken after 2 hr and analyzed for ethylene.

The effect of reducing endogenous ethylene on the wound response was studied using 5 subapical pea sections in 3-ml syringes. Before wound ethylene production started, endogenous ethylene was removed by vacuum flushing the tissue with ethylene-free air. The plunger was put into the syringe and inserted to the 0.5 ml mark. The syringe was closed and the plunger pulled back to the 3 ml mark (1/6 atm). The syringe contents were returned to atmospheric pressure by admitting ethylene-free air. The plunger was then depressed to the 0.5 ml mark; this procedure was repeated 10 times for each syringe. The same procedure was followed with the controls, except that the syringe contents were not subjected to subatmospheric pressure during flushing. The syringes were then set to 2.0 ml and capped with serum stoppers. After 30, 60 or 90 min, the plungers were reciprocated between the 1 and 3 ml marks about 10 times to equalize the internal and external concentrations of gases. Samples were taken and analyzed for ethylene, carbon dioxide and oxygen. The experiment was repeated 3 times with 5 replicates for each sample time.

Analysis of Data. Whenever possible, actual data from a representative experiment was used to study the kinetics of wound-induced ethylene synthesis. However, when there was a large amount of variability, curves were either fitted manually or drawn using data subjected to a 5-point curve-smoothing equation. The first peak of ethylene production was reasonably symmetrical. A second degree polynomial was fitted to data from the first peak by the method of least squares. The quadratic equation was then used to calculate the time of maximum ethylene evolution (the first derivative) and the amount of ethylene evolved during a standard interval about the maximum (the area under the curve as calculated from the integral equation, and bounded by the time of maximum ethylene production plus or minus 30 min). These parameters and similar data from accumulation studies were subjected to analysis of variance. Each experiment reported was repeated at least twice.

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Preliminary Observations. Ethylene production by 13-cm etiolated pea stems declined slightly after the apical region was cut, increased after about 25 min, and became significantly greater than the noninjured control after a lag-period of about 30 min (Fig. 1). Production by the noninjured control remained virtually constant during the experiment. Similar patterns were observed in three experiments. Two-hr accumulation experiments showed that 3 cuts perpendicular to the stem axis and half-way through the stem were as effective in eliciting wound ethylene (5.3 nl g^{-1} hr^{-1}) as were 3 cuts parallel to the stem axis (5.5 nl g^{-1} hr^{-1})

or 6 perpendicular cuts (5.4 nl g^{-1} hr^{-1}). The rates of ethylene production were not significantly different from each other, but were significantly different from the noninjured controls (3.9 nl g^{-1} hr^{-1}).

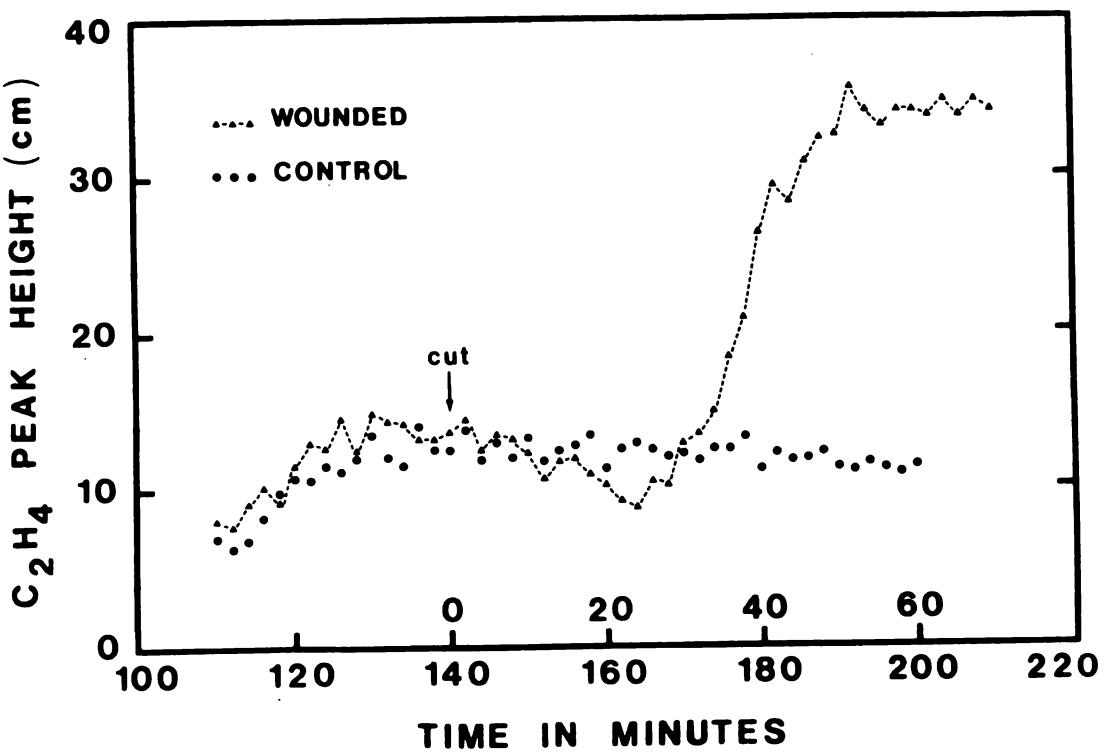

Figure 1. Ethylene evolution vs time from 13-cm etiolated pea stems which were kept in 26-ml sample chambers continually flushed with humidified ethylene-free air. Replicates were either left uninjured or wounded with 3 cuts perpendicular to the stem axis in the region 9 to 18 mm below the top of the apical hook.

Kinetics of Wound-Induced Ethylene Synthesis from Subapical Sections. Subapical stem segments were used to further characterize the pattern of wound ethylene synthesis. The 9-mm sections were excised from the same region that was wounded in the preliminary series of experiments. The trauma of excising the segments was sufficient to

elicit a characteristic pattern of wound ethylene production. Despite some variability, the major pattern of ethylene synthesis vs time at 25 C was clearly evident (Fig. 2). Averaging the min-by-min rates of

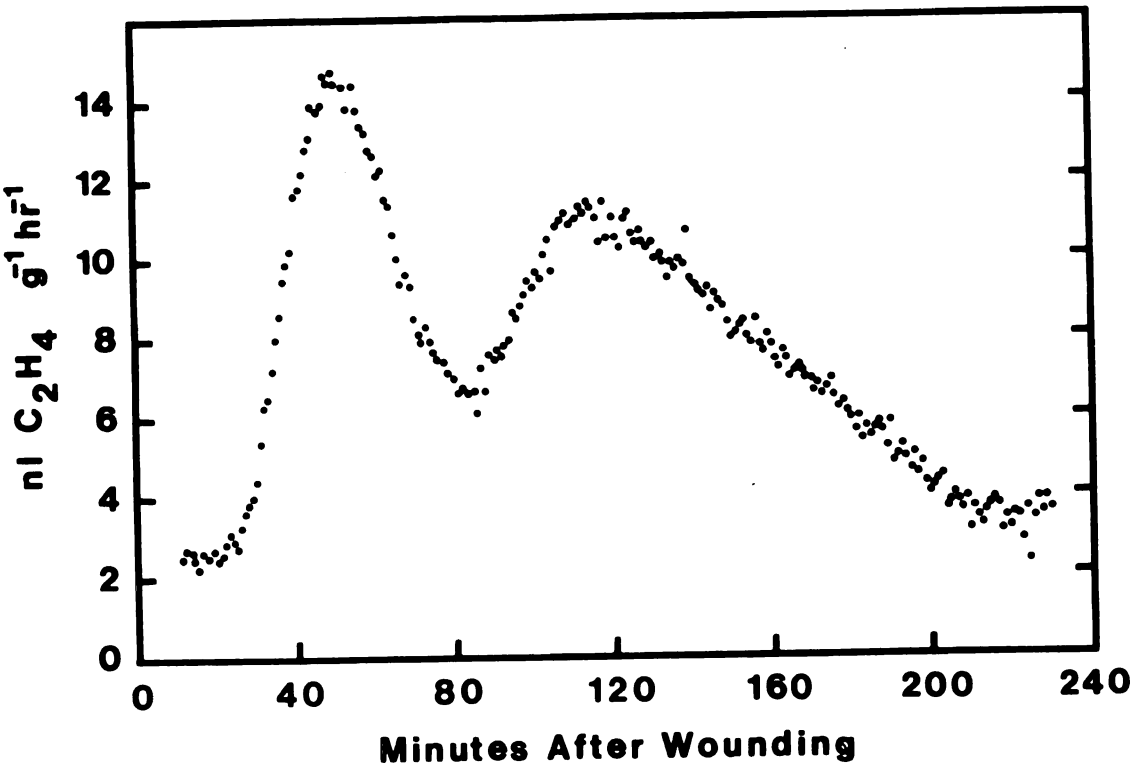

Figure 2. Ethylene evolution vs time from 9-mm subapical sections of etiolated pea stems held at 25 C. The sections were excised from the region 9 to 18 mm below the top of the apical hook.

ethylene synthesis from 12 experiments resulted in a remarkably consistent pattern (Fig. 3) Wound-induced ethylene synthesis commenced after a definite lag-period of 26 $\pm$ 1.4 min following excision of the subapical tissue segment. The rate of ethylene production increased linearly after this lag from 2.7 $\pm$ 0.3 nl g^{-1} hr^{-1} to the first maximum of 11.3 $\pm$ 1.8 nl g^{-1} hr^{-1} at 56 $\pm$ 2.1 min. The rate then decreased to a

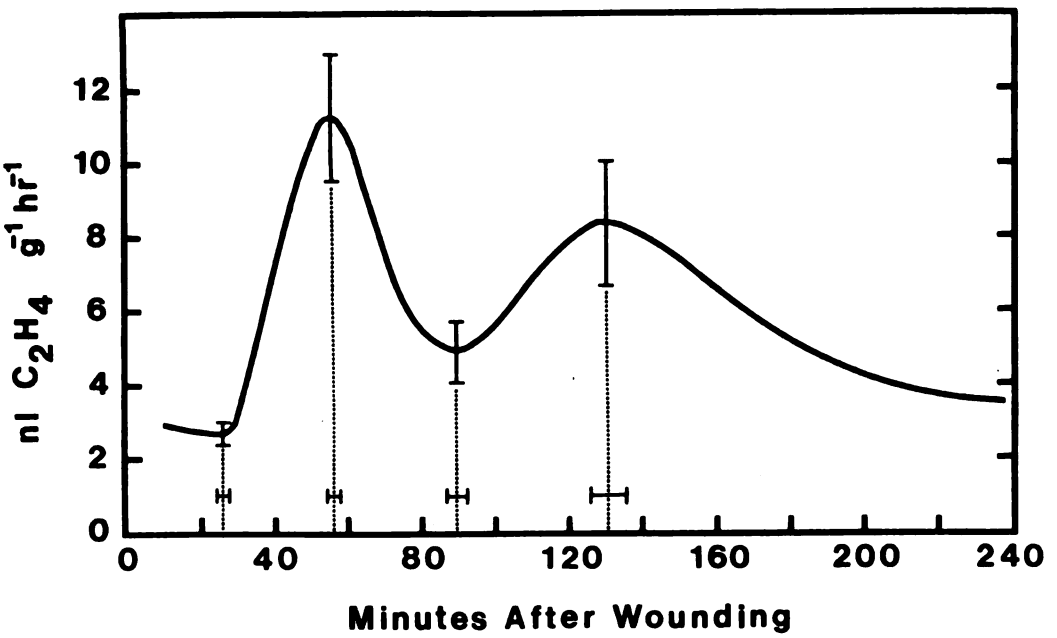

Figure 3. The kinetics of wound-induced ethylene synthesis from subapical pea stem sections. Data for this graph were taken from 12 representative experiments. The vertical lines denote the standard deviation of the rates of ethylene synthesis, while the horizontal lines denote the standard deviation of the times at which the inflection points occured.

minimum of 4.9 ± 0.9 nl g^{-1} hr^{-1} at 90 ± 2.7 min, increased to a reduced second maximum of 8.4 ± 1.7 nl g^{-1} hr^{-1} at 131 ± 4.9 min, and then declined over a period of about 100 min to a production rate of about 4.0 nl g^{-1} hr^{-1}.

Removal of endogenous ethylene by repeated evacuation and flushing of tissue sections with ethylene-free air did not significantly affect the timming or rate of wound ethylene synthesis. The characteristic pattern must therefore be the result of increased ethylene synthesis and not merely the result of facilitated diffusion of ethylene already present in the tissue through the newly exposed cut surfaces. This

conclusion is supported by the presence of a definite 26-min lag period. If the increase in ethylene evolution was due to enhanced diffusion a much more rapid increase would occur following cutting. This was tested by exposing subapical pea sections, in which the wound response had dissipated, to 10 ul l^{-1} ethylene for 15 min, then quickly flushing the sample chamber with ethylene-free air and following the subsequent evolution of ethylene from the tissue. A sampling rate of 1 ml min^{-1} caused an immediate exponential decrease in the rate of ethylene evolution which reached barely detectable levels after 7 min. This indicates that dissolved ethylene diffuses out of the tissue much too rapidly to be the source of the observed increase.

Effect of Various Factors. The length of time between harvesting a 13 cm etiolated pea stem and the excission of subapical section, attenuates subsequent wound ethylene production. Subapical sections from freshly harvested pea stems had a maximum rate of ethylene synthesis of about 11.3 nl g^{-1} hr^{-1} which occurred about 56 min after excision. If the cut 13 cm stems were stood vertically in water in a humid, aereated, ethylene-free atmosphere for 2 hr, the maximum rate of wound ethylene synthesis excised subapical segments was reduced to around 7.7 nl^{-1} hr^{-1}, which occurred about 55 min after excision. If the stems were held for 18 hr, no wound ethylene was observed from excised stem segments and the basal rate of ethylene evolution was barely detectable; ostensibly some factor dissipated with time.

Stem segments which had dissipated their initial wound response were not stimulated to produce a second response if rewounded. These aged sections still possess an ethylene synthesizing system since

treatment with 10 uM IAA results in similar increases in the rate of ethylene production by 18 hr, 1 hr, or freshly excised stem segments (Table 1).

Table 1. Effect of aging on wound- and IAA-induced ethylene production by subapical stem segments.

Time aged	C_2H_4 (nl g^{-1} hr^{-1})	
	Control	10 uM IAA
0 hr	4.4	8.8
1 hr	3.1	6.8
18 hr	2.7	5.6

The length of the internode from which the subapical sections were excised significantly affected both the time and the rate of maximum wound ethylene synthesis. As the internode length increased from 2 to 8 cm, the time of maximum synthesis shifted from 52 to 57 min. An increase in internode length from 6 to 8 cm caused a greater increase (1.8 min) than did an increase from 2 to 4 cm (1.1 min). The rate of maximum synthesis increased slowly from 5.5 nl g^{-1} hr^{-1} for sections excised from 2-cm internodes to 6.9 nl g^{-1} hr^{-1} for sections excised from 6-cm internodes, and then markedly increased to 8.2 nl g^{-1} hr^{-1} for sections excised from 8-cm internodes.

It was observed that etiolated peas form the fourth node when the

third internode was about 8 cm long. The great increase in wound ethylene synthesis from subapical sections excised from 8-cm internodes may reflect some morphological or physiological change in the stem. Tissue excised closer to the meristematic regions of the hook produced more wound-induced ethylene than tissue from differentiated regions. Extension of the meristematic region farther from the apical hook could account for the increased wound response, as could accumulation of substrates near the apex.

Changing the gravity orientation of excised subapical sections by rotating the sample chamber through 90 or 180 degrees after the onset of wound ethylene synthesis did not significantly affect the rate of ethylene production during a subsequent half-hour. However, apical sections oriented in their normal vertical position had significantly higher rates of ethylene synthesis (12.3 ± 1.8 nl g^{-1} hr^{-1}) than did sections oriented at random (9.0 ± 1.3 nl g^{-1} hr^{-1}). The lag-time and the rate of increase were identical for both treatments.

Exposure to fluorescent light had a slightly inhibitory affect on wound-induced ethylene. The timing was not changed, but exposure to light did reduce the maximum rate of ethylene synthesis by about 18%, from 10.3 ± 1.7 nl g^{-1} hr^{-1} in the dark, to 8.4 ± 1.5 nl g^{-1} hr^{-1} in the light.

Wound-Induced Ethylene Synthesis from Various Tissues. Different regions of etiolated pea plants were tested for their ability to produce wound-induced ethylene. Sections excised from meristematic regions of the stem or root exhibited high rates and multiple peaks of wound ethylene (Fig. 4). Tissue excised from different regions near the stem apex showed similar lag-times, rates of increase and maximum ethylene

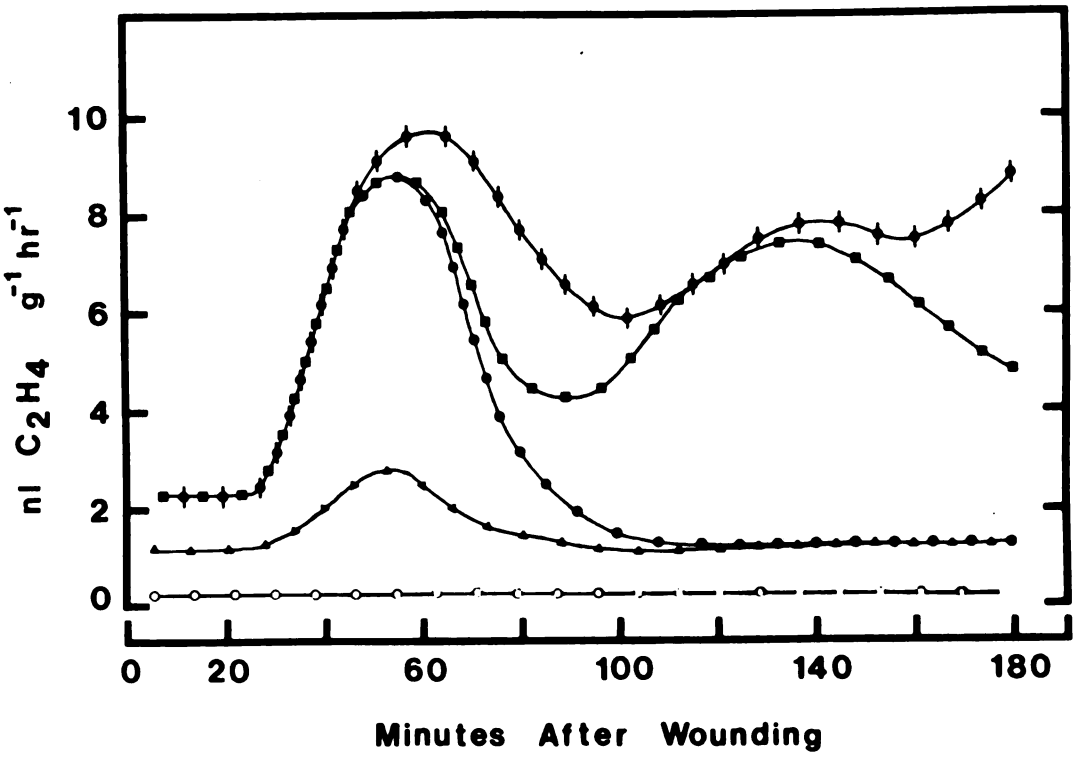

Figure 4. Ethylene synthesis vs time for various 9-mm segments of tissue from 7-day-old etiolated pea seedlings. Stem segments were excised from the region 0 to 9 mm (◆—◆), 9 to 18 mm (■—■), 13 to 22 mm (●—●), and 18 to 27 mm (▲—▲) from the top of the apical hook. Sections were also excised from the center of the second internode (○—○).

synthesis. As sections were excised progressively farther from the stem apex, the second peak diminshed and disappeared before the magnitude of the first peak was significantly affected. Segments excised still farther from the stem apex had lower rates of maximum ethylene synthesis during the first peak, but neither the lag-time nor the time of maximum synthesis was affected. Fully elongated stem or root tissue did not produce measurable amounts of wound-induced ethylene.

A wound-induced increase in the rate of ethylene production occurred in tissue excised from many different species of plants (Table 2). Segments from etiolated herbaceous plants had similar kinetics of

Table 2. Wound-induced ethylene synthesis from various species of plants.

Species	Tissue	Etiolated	Time (min) lag	Time (min) peak	Maximum rate (nl g^{-1} hr^{-1})
Pisum sativum	sub-apical	yes	26	55	10.6
Cucumis sativus	cotyledons	yes	24	57	3.7
Cucumis sativus	hypocotyl	yes	19	55	5.3
Lycopersicon esculentum	apical	yes	18	39	5.3
Hordeum vulgare	coleoptile	yes	24	47	22.4
Zea mays	coleoptile	yes	20	58	28.7
Zea mays	leaves	yes	20	58	60.3
Triticum aestivum	coleoptile	yes	18	53	29.6
Avena sativa	coleoptile	yes	16	50	30.7
Cercis canadensis	sub-apical	no	22	104	6.9
Forsythia viridissima	sub-apical	no	30	133	17.0
Lonicera flexuosa	sub-apical	no	55	129	1.2
Taxus canadensis	sub-apical	no	no wound-induced ethylene		

wound ethylene synthesis, while tissue from green woody plants were more variable in the timing and rates of ethylene production. Tissue from etiolated plants had similar lag-times (20 $\pm$ 3 min) and times of ethylene synthesis (53 $\pm$ 5 min). The lag-time for green tissue segments was variable, ranging from 22 min for Cercis canadensis to 55 min for Lonicera flexuosa. Taxus canadensis did not exhibit wound ethylene synthesis. The time to the maximum was over twice as long for green tissue as for etiolated tissue; ranging from 104 min for C. canadensis to 133 min for Forsythia viridissima. However, these differences may reflect specie differences, since etiolated vs non-etiolated tissues from one specie were not compared.

Apical coleoptile segments from etiolated monocots had much higher rates of wound ethylene synthesis (28 $\pm$ 3.7 nl g^{-1} hr^{-1}) than did stem tissue segments from etiolated dicots (6 $\pm$ 3.0 nl g^{-1} hr^{-1}). Excised leaves from 5-day-old etiolated Zea mays seedlings had an astonishingly high rate of ethylene synthesis (60 nl g^{-1} hr^{-1}).

Effect of Wounding on Ethylene, Carbon Dioxide, and Ethane Production. Since cutting fruits and vegetables stimulates the rate of respiration, as measured by oxygen uptake (20), wound ethylene might merely reflect fluctuations in the rate of respiration. However, the rate of respiration from 13 cm apical pea stems, as measured by CO_2 production, was only increased by 13%, from 509 ul g^{-1} hr^{-1} before wounding to 597 ul g^{-1} hr^{-1} 50 min after wounding, while wound ethylene production increased by 300%, from 2.6 to 10.4 nl g^{-1} hr^{-1}, in the same interval. Similarly, the rate of ethylene production by subapical stem segments increased almost 4 times from 4 nl g^{-1} hr^{-1} during the lag-phase to a maximum of around 15 nl g^{-1} hr^{-1}. During this same period

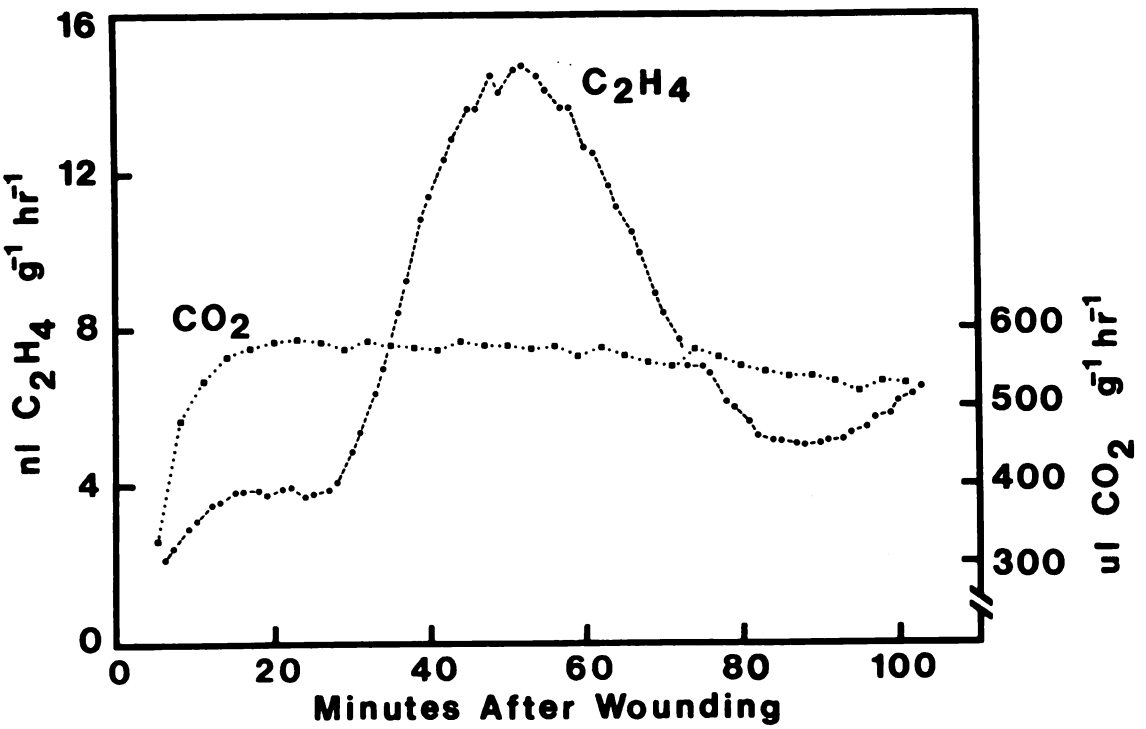

Figure 5. Ethylene and carbon dioxide evolution vs time from 9-mm subapical sections of etiolated pea stems.

the rate of carbon dioxide production, after an initial increase, remained constant at around 580 ul g^{-1} hr^{-1} (Fig. 5). Ethylene production fluctuated greatly during the first 100 min while carbon dioxide production showed a slight and almost linear decrease from 580 ul g^{-1} hr^{-1} to around 530 ul g^{-1} hr^{-1}. It should be noted that the rate of carbon dioxide production was over 4 orders of magnitude greater than the rate of ethylene production. Analysis of data from 9 experiments showed no consistently significant correlation between ethylene and carbon dioxide production during the first peak of wound-induced ethylene synthesis.

Ethylene and ethane production were followed for 3 hr following

excision of the sections. In 3 experiments the rate of ethane production did not change significantly as ethylene production went through its characteristic wound-induced pattern (Fig. 6). Lieberman and Kunishi

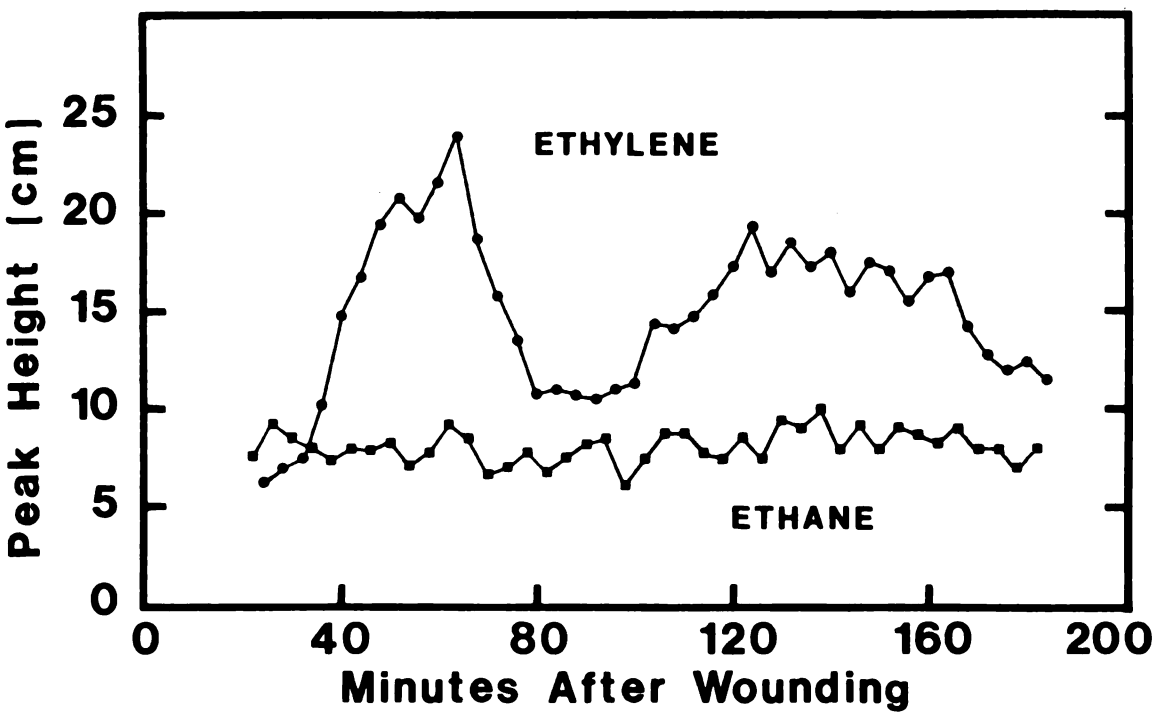

Figure 6. Ethylene and ethane evolution vs time from 9-mm subapical sections of etiolated pea stems.

(13) reported that in 2 model systems for ethylene synthesis from peroxidized linolenic acid, the ratio of ethylene to ethane production was about 15:1 and 4:1. The absence of a constant ratio between wound ethylene production and ethane evolution argues against a similar *in vivo* biosynthetic system for the production of wound ethylene from linolenic acid. Experiments with labeled linolenic acid (15), and its peroxidized product propanal (4), have shown that neither is converted to ethylene in living cells.

CONCLUSION

Mechanically wounded etiolated pea stem sections produce a remarkably consistent pattern of wound-induced ethylene synthesis. Variations in tissue preparation and execution of the experiment slightly perturb the magnitude, but did not markedly alter the timing of the response. A definite lag-period of 26 min precedes the induction of 1 or 2 well-defined surges of ethylene synthesis which occur at 56 and 131 min. This is a rapid, stable, and predictable system with which to study the physiology of wound-induced ethylene synthesis.

In the next paper of this series, the O_2 and temperature dependency and the initiation of the response are investigated.

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SECTION TWO

STUDIES OF RAPIDLY INDUCED WOUND ETHYLENE SYNTHESIS BY EXCISED SECTIONS OF ETIOLATED PISUM SATIVUM L., CV. ALASKA

II. OXYGEN AND TEMPERATURE DEPENDENCY, AND INDUCTION OF THE WOUND RESPONSE

OXYGEN AND TEMPERATURE DEPENDENCY, AND INDUCTION OF THE WOUND RESPONSE

ABSTRACT

Earlier studies (see section one) established that excision of subapical stem sections from etiolated seedlings of Pisum sativum L., cv. Alaska, was sufficient injury to elicit a characteristic pattern of ethylene synthesis. At 25 C, wound ethylene synthesis increased linearly after a 26 min lag period, from 2.7 nl g^{-1} hr^{-1} to the first maximum of 11.3 nl g^{-1} hr^{-1} at 56 min. The rate of production decreased to a minimum at 90 min, increased to a lower second maximum at 131 min, and subsequently declined over a period of 100 min to about 4.0 nl g^{-1} hr^{-1}.

Wound-induced ethylene synthesis during the first 90 min was half-saturated at 3.6% O_2, while CO_2 production was half-saturated at 1.06% O_2. Both reactions were saturated at about 10% O_2. Anaerobiosis stopped all ethylene synthesis and delayed the characteristic pattern of wound ethylene synthesis by the length of time freshly excised tissue were deprived of O_2. However, once the rise in wound ethylene synthesis had started, a period of anaerobiosis altered the normal pattern of wound ethylene synthesis when the tissue were returned to air.

Exogenously applied ethylene, or its analogue propylene, stopped wound ethylene synthesis during the time it was applied, but permitted induction of the wound response since wound ethylene synthesis started to rise immediately after the tissue were flushed with air. Lowering

the internal concentration of ethylene, by enhanced diffusion at low pressure (130 mm Hg), almost doubled wound ethylene synthesis. Addition of propylene negated the effect of low pressure. Exposure to 4% CO_2 in air reduced wound ethylene production by 30%. It was concluded that the decline in wound ethylene synthesis, which produced the minimum at 90 min, resulted from negative feedback control of endogenous ethylene on wound ethylene synthesis.

No wound ethylene was produced during the 2 hr after excision at temperatures above 36 C or at 10 C. The low temperature allowed induction of the wound response since tissue immediately produced wound ethylene when warmed to 30 C after 2 hr at 10 C. Temperatures above 36 C stopped wound ethylene synthesis by more drastic means since tissue cooled to 30 C after 1 hr at 40 C required 2 hr before ethylene production returned to normal levels. An Arrhenius plot showed two abrupt changes in the activation energy: one at 15 C and the other at 36 C. The activation energy between these temperatures was 12.1 Kcal $mole^{-1}$ $degree^{-1}$.

The subapical region of etiolated 'Alaska' pea seedlings was induced to synthesize wound ethylene by cuts made over 2 cm away, while excision of subapical sections induced wound ethylene synthesis throughout the length of 9- or 16-mm sections. Accumulation of a substance at the cut surface of excised sections, as the result of interrupted translocation, did not initiate or significantly contribute to wound ethylene synthesis. Cutting subapical sections into smaller pieces showed that there are two zones of wound ethylene synthesis. Cells less than 2 mm from the cut surface produced about 30% less wound ethylene than cells greater than 2 mm from the cut surface.

INTRODUCTION

In a previous study (see section one) a rapidly induced, transitory increase in ethylene synthesis was shown to occur in tissue excised from the actively growing regions of etiolated barley (Hordeum vulgare L., cv. Maraina), cucumber (Cucumis sativus L., cv. Pioneer), maize (Zea mays L., cv. D-5), oat (Avena sativa L., cv. Victory), pea (Pisum sativum L., cv. Alaska), tomato (Lycopersicon esculentum Mill., cv. Campbell 28), and wheat (Triticum aestivum L., cv. Ionia) seedlings. The kinetics of wound-induced ethylene synthesis varied with the region of the plant from which the tissue segment was excised. The kinetics were characterized for 9-mm subapical stem segments excised 9 mm from the top of the apical hook, from etiolated 7-day-old seedlings of Pisum sativum L., cv. Alaska. Wound-induced ethylene production at 25 C started to increase linearly after a lag period of 26 min from 2.7 nl g^{-1} hr^{-1} to the first maximum of 11.3 nl g^{-1} hr^{-1} at 56 min. The rate of production then decreased to a minimum at 90 min, increased to a lower second maximum at 131 min, and then declined over a period of about 100 min to about 4.0 nl g^{-1} hr^{-1}. Variations in tissue preparation and execution of the experiment slightly perturbed the magnitude, but did not markedly alter the timing of the response.

Vegetative, ripening, and senescent tissue require O_2 for ethylene synthesis (3,8,11,20,45,56). Sfakiotakis and Dilley (49) found that induction of autocatalytic ethylene production by apples occurred above 6.5% O_2 and that anaerobiosis extinguished induced ethylene production. Lieberman and Spurr (33) showed that ethylene synthesis by broccoli (Brassica oleracea L.) was prevented at 0.0% O_2 and saturated at between

1.0 and 2.5% 0_2. Mature green banana fruit (Musa spp.) maintain normal, though reduced, basal respiration at an 0_2 tension of 5.0%, but are unable to produce ethylene in atmospheres of less than 7.5% 0_2 (38). Burg and Thimann (15) showed that ethylene production by plugs of climacteric apples (Malus domestica Borkh.) decreased at around 10% 0_2 and had a half maximal rate at around 2.0% 0_2. They suggest that since apples show a strong Pasteur effect (i.e. enhanced glycolysis at 0_2 tensions at or approaching zero), the cessation of ethylene synthesis in the absence of 0_2 is not merely the result of insufficient metabolic energy. Similarly, the induction of the Pasteur effect in anaerobic peas (5,52) should eliminate reduced metabolic energy as the cause of reduced wound ethylene synthesis at low 0_2 tensions.

In a current review on ethylene biosynthesis, Yang (59) postulates that ethylene may be formed from an unidentified intermediate between methionine and ethylene by an 0_2-dependent reaction. Support for this hypothesis comes from the observation that there is a surge of ethylene production when anaerobic pear (20) or apple (8,15) tissue is returned to air. Since the 0_2-dependent reaction could not function under anaerobic conditions, the concentration of the proposed intermediate would build up at the site of synthesis so that increased ethylene production would occur upon exposure to 0_2 before equilibrium was re-established.

Cutting tissue results in an immediate increase in the rate of 0_2 uptake (27,34,37,47) which has been associated with induction of the cyanide-insensitive respiratory pathway (34) and enhanced glycolysis. Laties (29) showed that the tricarboxylic acid cycle is blocked between citrate and α-ketoglutarate oxidation in freshly cut potato disks. Pea seedlings and their mitochondria are slightly cyanide-sensitive (21,53).

The rate of ethylene production increased in plants which had been frozen (55,57,60), given a cold stress of 5 C (16,50), or a heat stress of 40 C (25), and decreased in plants exposed to 40 C (15,17,20,32,40). Ethylene production by apples reached a maximum at 32 C and declined to zero as the temperature rose to 40 C (15). Production recovered slowly when the temperature was lowered to 22 C, reaching about 50% of the normal 22 C rate in 5 hr. A similar recovery occurred in heat stressed pears (20). Availability of substrate is not the limiting factor since ethylene production by apple tissue fed methionine was also severely inhibited at 40 C (32). In general, ethylene production is reduced at temperatures above 35 C and extinguished by 40 C (3,10). Arrhenius plots of the respiration rate of chilling resistant plants (e.g. tomato fruit, cucumber fruit, and sweet potato roots) show an abrupt decrease in the log of the respiration rate at 9 to 12 C (35). Abrupt changes in the activation energy of respiration and other processes have been associated with phase changes in structural or enzymatic components (for a thorough discussion, see ref. 48).

If the level of stress is sufficiently severe, physiological ethylene production is no longer stimulated by increased stress, but is diminished or completely abolished (2,44,58). Ethylene synthesis was stimulated by cutting apples (43), bananas (41), tomatoes (31), sweet potato roots (22), bean petioles (24), and etiolated rice coleoptiles (23) into pieces. However, cutting the tissue into smaller and smaller pieces, or homogenizing the tissue, reduced or stopped all ethylene production (22,31,43). Jackson and Osborne (24) reported that cutting 2 cm petiole segments of Phaseolus vulgaris L., cv. Canadian Wonder into smaller pieces stimulated ethylene production in proportion to the number

of cut surfaces. Increasing the cut surface area of sweet potato roots (Ipomoea batatas L., cv. Norin 1) stimulated ethylene production in proportion to the logarithm of the area (22). Imaseki et al., (23) reported that cutting etiolated rice (Oryza sativa L., cv. Aichi-Asahi) coleoptiles into smaller segments stimulated ethylene production, but that the increase was not proportional to the number of cut surfaces.

Elstner and Konze (19) showed that ethylene and ethane production from sugar beet (Beta vulgaris L.) leaf disks increased linearly until 50% of the leaf disk had been point frozen by a 3 mm diameter stainless steel rod kept at the temperature of liquid nitrogen. Freezing any additional area resulted in a linear decrease in ethylene production, but continued the linear increase in ethane production. They suggested that the frozen, i.e. killed, cells produce ethane, while cells near the frozen spots were perturbed and produced ethylene. Considering their data and the geometry of their experiment, it can easily be calculated that cells up to about 0.6 mm from the edge of the frozen spot were perturbed. With this configuration, when 50% of the leaf disk had been point frozen all of the remaining area would have been perturbed and would be producing ethylene. The next spot frozen would remove that area from ethylene production and convert it to ethane production, thereby producing the observed decrease in ethylene production while maintaining the increase in ethane production. This compares to an activated zone of less than 3 mm from the cut surface of petiole sections (24) and 0.6 mm from the edge of frozen sugar beet leaf cells (19). Laties (28) reported that increased respiration occurs initially throughout a potato tuber slice, and that the duration of the rise in any part of the slice is dependent on the distance from the cut surface.

This study further characterized wound-induced ethylene synthesis by etiolated 'Alaska' pea stem segments and its O_2 and temperature dependency, and induction of the characteristic pattern of wound ethylene evolution.

MATERIALS AND METHODS

Plant Material. Seven-day-old etiolated seedlings of 'Alaska' pea were grown, and prepared as previously described (see section one). Apical stem segments included the apical meristem, plumule, apical hook, and a portion of the stem. They were cut to lengths of 13 cm or 27 mm from the top of the apical hook. All kinetic studies of wound-induced ethylene synthesis were done with 9-mm subapical stem sections excised 9 mm from the top of the apical hook. Some experiments use tissue which had dissipated their initial wound response (aged sections). The procedures for identifying and quantifying ethylene, CO_2, and O_2 were the same as previously described (see section one).

Oxygen Dependency. The O_2 requirement for wound-induced ethylene synthesis was studied using 20 subapical sections enclosed in 25-ml Erlenmeyer flasks with moist filter paper and a CO_2 absorber. The flasks were purged with N_2 to remove all O_2, and an appropriate volume of air or pure O_2 was injected by syringe to give a specified O_2 concentration. After 2 hr the gaseous contents of the flasks were sampled and analyzed for ethylene, O_2 and CO_2.

Endogenous Ethylene Effect. Vacuum removal of endogenous gases from aged sections was accomplished in the flow-through-system by inserting the needle of a 50-ml syringe through the top serum stopper of an

0.8 ml opaque, glass sample chamber. A vacuum was created by closing the gas inlet tube to the bottom of the sample chamber and pulling the plunger of the syringe to the 50 ml mark. After a few sec the inlet tube was opened and ethylene-free air entered the evacuated chamber. The syringe was withdrawn when the flow into the chamber stopped, and sampling was resumed.

The effect of lowering the concentration of endogenous gases, by enhancing diffusion at low pressure, on wound ethylene production was studied in a static system using 10 subapical sections held at 760 mm or 130 mm Hg. The 30-ml syringes were flushed with ethylene-free air or 100% O_2, set to 4 ml, capped with rubber serum stoppers, and 1 ml of air, 100% O_2, 0.15% CO_2, or 250 or 1500 ul liter^{-1} propylene injected through the serum stopper. The plungers were left at 5 ml or pulled to the 30 ml mark and held with restraints. No leakage of gas occurred in the low pressure syringes. After 120 min the restraints were released and the contents of all syringes analyzed for ethylene and CO_2.

Temperature Dependency. The effect of temperature on wound-induced ethylene synthesis was studied using a flow-through-system in which 7 subapical sections were enclosed in 0.8 ml opaque, glass sample chambers immersed in water baths. The flow-through-system allowed 2 ml samples to be taken from each sample chamber. Three chambers were run in parallel with sampling on a staggered 40 sec schedule: i.e. I sampled at 0.0 sec, II sampled at 40 sec, III sampled at 80 sec, I sampled again at 120 sec, etc.). This gave a flow rate of 60 ml hr^{-1} which produced concentrations of ethylene easily detected by the gas chromatograph. Preliminary experiments showed that running parallel samples reduced variations in the kinetics of wound-induced ethylene synthesis resulting from the order

in which the subapical stem sections were prepared during the day. Each 2 ml sample removed over 90% of the gas in the chamber. Since each sample contained almost all the ethylene evolved during the 2 min collection period, and since the gas chromatograph responds to the amount of ethylene in a sample, and not its concentration, the rate of ethylene synthesis was caluclated from the chromatographic output without correcting for temperature-caused volume changes in the samples (i.e. the STP gas volume would be different for a 2 ml sample at 10 than at 40 C; however, if each sample contained 1.0 pico moles of ethylene the chromatographic output would be the same). The rate of synthesis was therefore calculated as pico moles (10^{-12} moles) per gram fresh weight hr (p moles g^{-1} hr^{-1}). Later more accurate measurements were made using 30 subapical sections enclosed in a 1.7 ml opaque, glass sample chamber with a thermometer (0.01 C divisions) inserted through the top serum stopper and in contact with the tissue. Direct readings of the tissue temperature were made, rather than inferring the temperature from that of the water bath. Two ml gas samples were analyzed for ethylene every min.

Transmission of a Wound Signal. The ability of a wound signal to be transmitted through the stem was investigated using 9-mm subapical sections excised from 13 cm or 27 mm apical stem segments freshly harvested or held in a humid, ethylene-free atmosphere for 15 min. Subapical sections were excised and the kinetics of wound ethylene evolution studied for 90 min.

Development of polarity in wound-induced ethylene synthesis was studied with subapical sections left whole or cut into apical and basal halves at 0, 15, 30, 60, or 90 min after excision. The tissue was

enclosed in 25-ml Erlenmeyer flasks containing moist filter paper and a CO_2 absorber. Gas samples were analyzed for ethylene 30 min after time zero, and at 60 min after all other times.

Induction of wound ethylene synthesis by accumulation of a translocatable substance at the cut surface was studied by removing 3 mm from the apical or basal end of a 16-mm subapical section. The 16-mm section was excised 6 mm from the top of the apical hook, so that after removal of the two 3 mm sections of stem tissue, the 10-mm subapical section would contain similar tissue to that in the normal 9-mm subapical section. Apical stem segments were excised and attached to 3 X 5 inch index cards with 2-sided adhesive tape. The cards had been marked to permit rapid and accurate cutting of the tissue. The cards were maintained in a vertical position in a humid, ethylene-free atmosphere. Sections were cut to the required 16 mm length at time zero and trimmed of the apical and/or basal 3 mm's at either zero time or after 15 min. The four treatments were therefore cut at (apical 3 mm removed/basal 3 mm removed) zero/zero, zero/15 min, 15 min/zero, 15 min/15 min. Twenty min after excision of the 16-mm subapical section, the trimmed sections, which were now 10 mm in length, were transferred to 0.8 ml opaque, glass sample chambers and the kinetics of wound ethylene evolution were followed for 90 min.

The effect of additional wounding was studied by cutting 9-mm subapical sections into halves, thirds, fourths, or sixths. The tissue was placed in 0.8 ml opaque, glass sample chambers as soon as excised and subdivided, and the kinetics of ethylene evolution were followed for 90 min.

Analysis of Data. Whenever possible, actual data from a

representative experiment were used to study the kinetics of wound-induced ethylene synthesis. To more accurately determine the time of maximum wound ethylene production, and the amount of ethylene evolved during a certain interval, a second degree polynomial was fitted by the method of least squares to data from the first peak. Preliminary calculations showed that the first peak was very symmetric, so that a curve fitted by this method would provide valid data. The quadratic equation was used to calculate the rate and time (i) of maximum ethylene evolution (using the first derivative), and the amount of ethylene evolved during a standard interval ($i \pm 30$ min) about the time of maximum ethylene synthesis (the area under the curve as calculated from the integral equation, and bounded by the time of maximum ethylene synthesis plus or minus 30 min). These parameters, similar data from accumulation studies, and other kinetic data were subjected to analysis of variance. Each experiment reported was repeated at least twice.

Since most experiments were run at 24 C, the rate of ethylene synthesis can be converted from p moles g^{-1} hr^{-1} to the more common nl g^{-1} hr^{-1} by multiplying by 0.024.

RESULTS AND DISCUSSION

Oxygen Dependency. Wound-induced ethylene synthesis was reduced at O_2 concentrations below 10% (v/v) and followed a general $1\text{-}e^{-kx}$ decline as the O_2 concentration approached zero (Fig. 7). The rectangular hyperbolic curve fitted to the data demonstrates that the rate of wound ethylene synthesis is proportional to O_2 at low O_2 concentrations, and that above 10% O_2 the reaction producing wound ethylene becomes

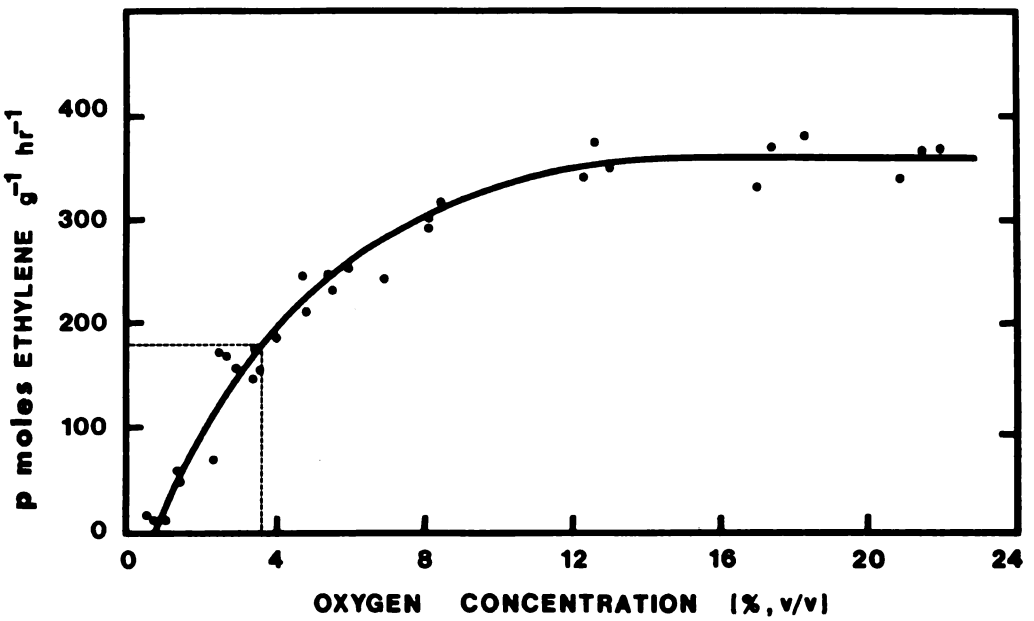

Figure 7. Effect of oxygen concentration on 90 min accumulation of wound-induced ethylene synthesis by 9-mm subapical stem sections of etiolated 'Alaska' pea seedlings.

saturated and independent of the O_2 concentration. The half-maximal rate of wound ethylene synthesis occurred at around 3.6% O_2. This concentration was higher than the half-maximal concentration reported for broccoli, but similar to those for apples and bananas (15,33,38). Wound ethylene synthesis was undetectable (less than 1 nl liter^{-1}) in flow-through-systems in which the O_2 concentration was maintained below 0.01%. However, the lowest O_2 concentration obtained in the 90 min accumulation experiments was around 0.5% O_2. The intersection of the fitted curve with the abscissa at ca. 0.7% O_2 suggests that an O_2 threshold may exist for wound ethylene synthesis.

CO_2 production was half-saturated at 1.06% O_2 and saturated at around 10% O_2. In a variety of excised tissue, respiration has been reported to be rarely saturated at O_2 concentrations below 10%, and to be half-saturated at around 2 to 4% O_2 (4,9). The difference between

the half maximum 0_2 concentration for respiration (1.06%) and for wound ethylene biosynthesis (3.6%) could result from competition for 0_2 by various other metabolic reactions.

The characteristic pattern of wound-induced ethylene synthesis was delayed by the length of time freshly prepared tissue was deprived of 0_2 (Fig. 8). The length of the lag period and the time to maximum

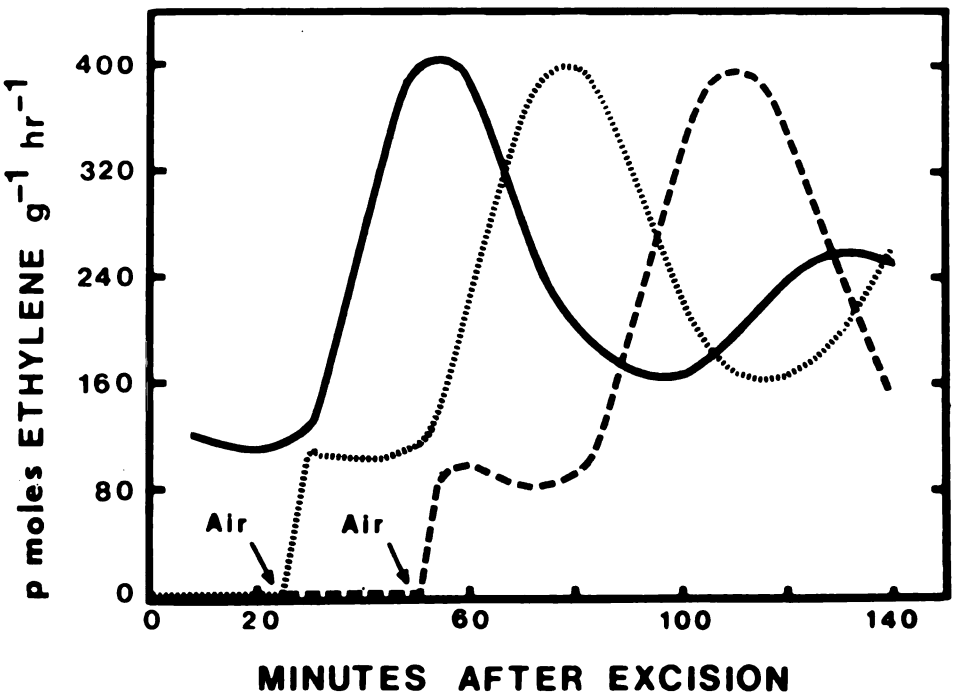

Figure 8. Effect of anaerobiosis on wound-induced ethylene synthesis by freshly excised 9-mm subapical sections from etiolated pea stems.

ethylene production were both increased in tissue held under N_2 for 25 or 50 min. However, the maximum rate of wound ethylene production was unaffected by the period of anaerobiosis. Apparently 0_2 is necessary for reactions during the lag phase which bring about the increase in ethylene synthesis. Interrupting the normal response, after its induction, with a 30 min period of anaerobiosis stopped ethylene production

during the time 0_2 was absent (Fig. 9). Admission of 0_2 reinstated the characteristic pattern at the point at which it was interrupted if the stoppage had occurred before 35 min; after this time anaerobiosis altered the normal pattern of wound-induced ethylene synthesis.

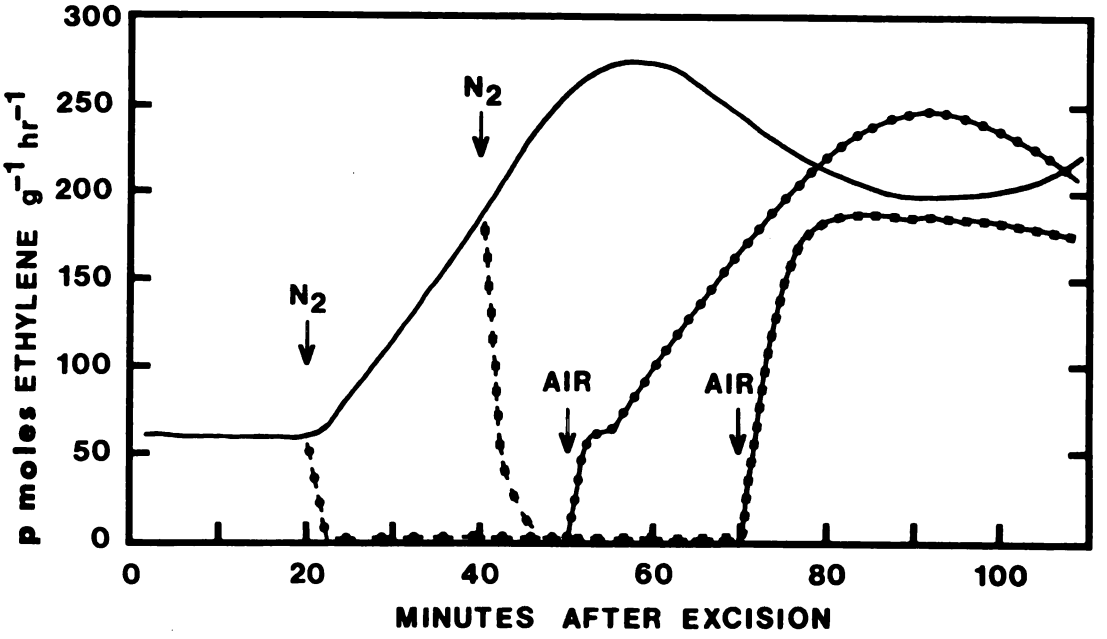

Figure 9. Effect of a 30 min period of anaerobiosis on wound-induced ethylene synthesis given before or after induction of increased synthesis. The tissue consisted of 9-mm subapical sections of etiolated pea stems.

Subapical pea stem segments which had dissipated their initial wound response and had been deprived of 0_2 for 1 to 3 hr showed a rapid increase in ethylene production, as soon as air was readmitted, to between 30 and 50% of the rate before anaerobiosis (Fig. 10). A slight decline followed before a second increase started about 35 min after exposure to 0_2. The rise continued to a peak at around 70 min which generally was about twice the original rate of ethylene production. Ethylene production then declined and stabilized near the original rate of production in about 1 hr. Although the exact timing is different,

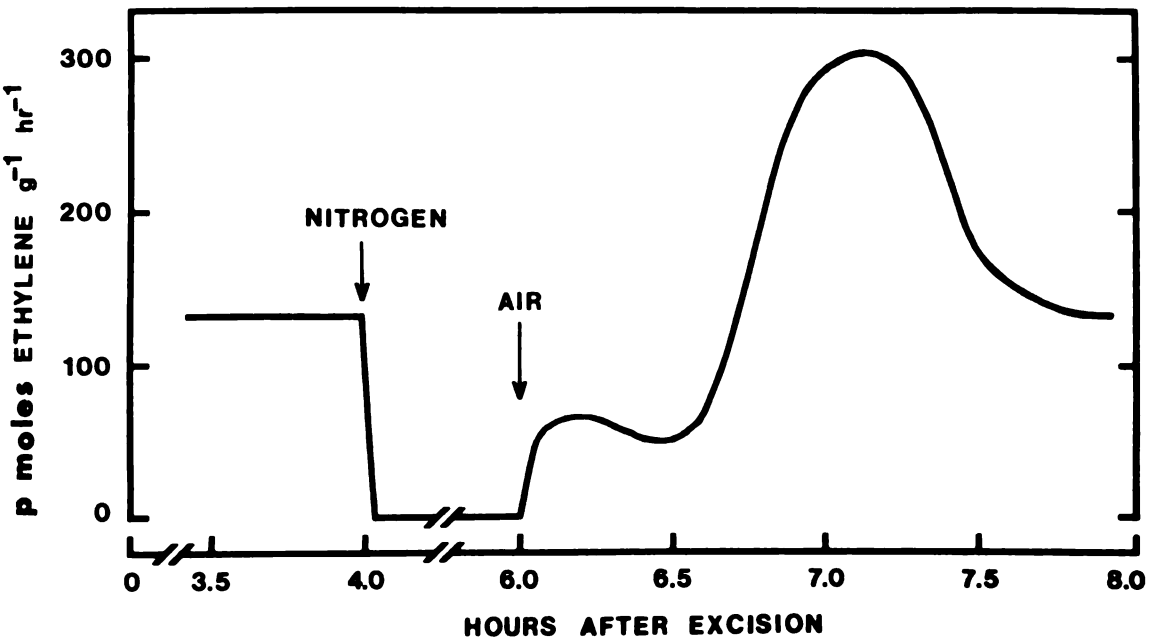

Figure 10. Effect of anaerobiosis on ethylene production by aged 9-mm sections of etiolated pea stems. The atmosphere was changed from air to nitrogen, and from nitrogen to air at the designated times.

the general pattern is similar to that of wound-induced ethylene synthesis. However, enhanced diffusion of O_2 into excised stem segments through the cut surfaces is not the cause of wound ethylene since, 1.) coating the cut ends of freshly excised tissue with silicon stopcock grease did not prevent the characteristic pattern of wound ethylene evolution, and 2.) gassing 13-cm apical stem segments with 100% O_2 did not induce a wound response or increase the rate of ethylene production. No surge in CO_2 production was observed following the change from N_2 to air. A burst of ethane sometimes accompanied the change from air to N_2 or N_2 to air. However, the results were not consistent.

Anaerobiosis gradually reduced the rate of CO_2 production by 50% in 2 hr, from 585 to 287 ul g^{-1} hr^{-1} from subapical stem segments. However, the energy yield of glycolysis is only 5% of aerobic respiration, so that

although a Pasteur effect was seen, this does not mean that a similar energy flux as ATP was produced (51). In the present study, wound ethylene production was reduced 50% when subapical sections were dipped in 0.5 mM KCN for 2 min. The rate of ethylene synthesis decreased from 2.6 nl g^{-1} hr^{-1} to 1.3 nl g^{-1} hr^{-1}, while the rate of CO_2 production was only reduced by 20%, from 590 ul g^{-1} hr^{-1} to 470 ul g^{-1} hr^{-1}. Palmer and Loughman (46) reported that the rate of respiration of etiolated pea epicotyls decreased following wounding. However, in this study it was observed that wounding increased respiration,as measured by CO_2 production, by 13%, from 509 ul g^{-1} hr^{-1} before wounding to 597 ul g^{-1} hr^{-1} 50 min after wounding, while in the same time interval, ethylene synthesis increased 300%, from 2.6 to 10.4 nl g^{-1} hr^{-1}.

Vacuum flushing subapical stem sections which had dissipated their initial wound response with air, resulted in an overshoot of ethylene production before the equilibrium rate of ethylene synthesis was re-established (Fig. 11). This suggests that basal, and wound-induced ethylene synthesis could be regulated by the amount of O_2 diffusing into the tissue, or by the internal concentration of ethylene or CO_2. These hypotheses were tested by varying the endogenous concentrations of ethylene, O_2, and CO_2 in freshly harvested subapical pea sections. If one of these gases exerts control on wound ethylene synthesis, then reducing its internal concentration should reduce the decline in wound ethylene production seen at 56 to 100 min. An 80% He: 20% O_2 (v/v) gas mixture increased gas diffusivity, but did not significantly affect the kinetics of wound ethylene synthesis. Pressures of 0.2 atm are often used to increase gas diffusivity to the extent that the internal concentration of ethylene is lowered below the threshold value needed to

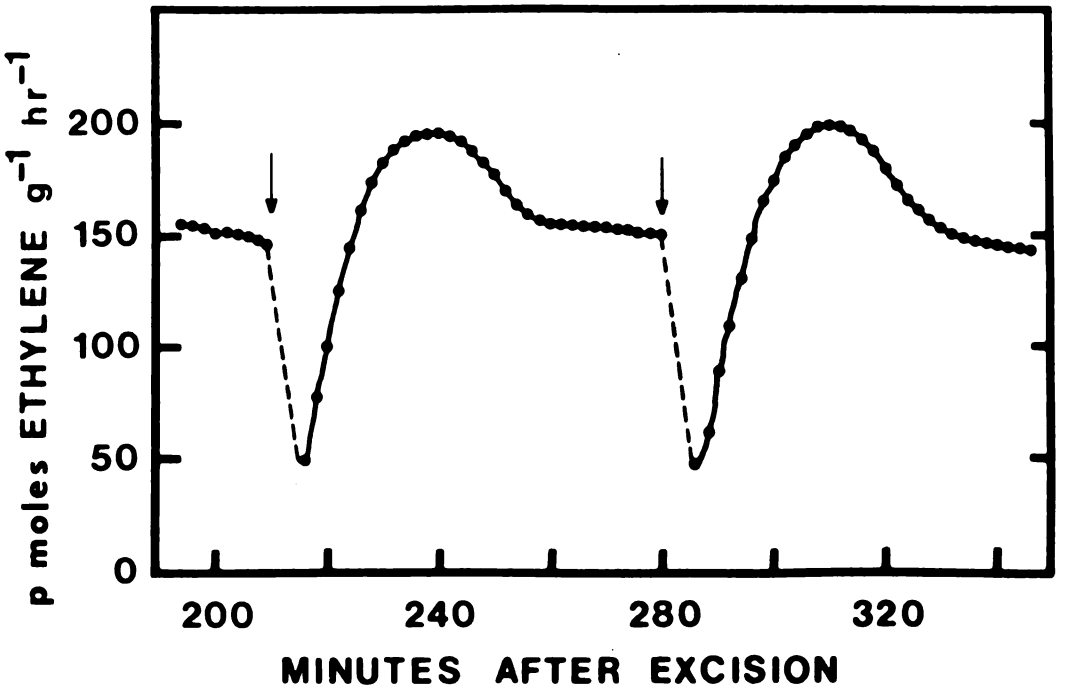

Figure 11. Effect of vacuum removal of endogenous gases on ethylene synthesis by subapical sections of etiolated 'Alaska' pea seedlings which have dissipated their initial wound response. The arrows represent a vacuum flushing with ethylene-free air.

ripen fruits and vegetables (13). Since a flow-through-system was not available which would permit sampling ethylene evolution at below atmospheric pressure, a series of experiments were performed using a static system.

Experiments conducted with partial pressures of around 20% O_2 at normal (760 mm Hg) or 1/6 atm (130 mm Hg) pressure showed that almost twice as much wound ethylene was produced at the reduced pressure (Table 3). Addition of CO_2 to give the normal partial pressure of 0.03% (i.e. 0.23 mm Hg) had no significant effect on wound ethylene synthesis. However, gassing subapical sections with 3.5% CO_2 in air in the flow-through-system, significantly reduced the rate of maximum wound ethylene synthesis from 12.3 $\pm$ 1.3 nl g^{-1} hr^{-1} in air, to 8.2 $\pm$ 1.5 nl g^{-1} hr^{-1},

Table 3. Ethylene and carbon dioxide accumulation by excised subapical 'Alaska' pea stem sections held at 760 or 130 mm Hg for 2 hr.

Partial pressure of gases (mm Hg)	C_2H_4 (p moles g^{-1} hr^{-1})		CO_2 (ml g^{-1} hr^{-1})	
	760 mm Hg	130 mm Hg	760 mm Hg	130 mm Hg
760 mm O_2	218	---	1.07	----
130 mm O_2	178	351	0.89	1.13
+ 0.23 mm CO_2	198	336	0.87	1.11
+ 250 ul $liter^{-1}$ C_3H_6	112	149	0.93	1.16

and the accumulation during the interval i $\pm$ 30 min from 8.9 $\pm$ 0.8 nl in air to 6.4 $\pm$ 0.9 nl. The time of maximum wound ethylene synthesis was not significantly affected. The 30% reduction in ethylene synthesis shows that CO_2 is an inhibitor of wound ethylene synthesis, as it is in other systems where it is thought to act as a competitive inhibitor of ethylene action (3,12). Ethylene production by climacteric fruit is reduced by the competitive inhibition of CO_2 with endogenous ethylene for ethylene receptor sites (14). If CO_2 controls wound ethylene synthesis by competing with endogenous ethylene for positive feedback sites, then fluctuations in the internal concentration of CO_2 could produce the characteristic pattern of wound-induced ethylene evolution. However, previous work has shown that during the first 2 hr, CO_2 production remains almost constant as wound ethylene production fluctuates over 400% (see section one).

Addition of the ethylene analogue propylene (14,42) significantly reduced wound ethylene production at normal and low pressure. Ethylene has been shown to inhibit ethylene production by green tomato fruit and

non-ripening stages of fig fruit (Ficus sycomorus L., cv. Balami)(61) and by slices of green banana fruit (54). Propylene has a similar effect in these systems, but competes with ethylene in other systems. Propylene has been shown to compete with ethylene in its ability to increase stem thickness, reduce stem growth, and cause diageotropism in etiolated 'Alaska' pea seedlings (18). Propylene may therefore not be an analogue of ethylene, but like CO_2, a competitive inhibitor of ethylene action in this system. Reduction of wound-induced ethylene synthesis may be the result of competition between ethylene and propylene for positive feedback sites, rather than propylene augmenting activation by ethylene of a negative feedback site. This question was resolved by gassing freshly excised subapical sections with ethylene or propylene.

The initial peak of wound-induced ethylene synthesis was delayed by exposure to either 11 ul $liter^{-1}$ ethylene or to a physiological equivalent amount (500 ul $liter^{-1}$) of propylene (Fig. 12). The rate of wound ethylene production under 500 ul $liter^{-1}$ propylene was only slightly less than the rate during the normal lag period or from the stable rate of production from non-wounded apical regions of pea stems (see section one). When these sections were returned to air, it was observed that exposure to either ethylene or propylene had affected the kinetics of wound ethylene synthesis; the rate of production during the first peak was reduced, and the peak broadened. Gassing with ethylene or propylene for 1 hr eliminated the 26 min lag period seen in freshly excised tissue. Ethylene and propylene did not stimulate wound ethylene synthesis since no wound increase in ethylene production was observed while gassing with propylene, and the initial rate of ethylene production was very low when these sections were returned to air. It appears that

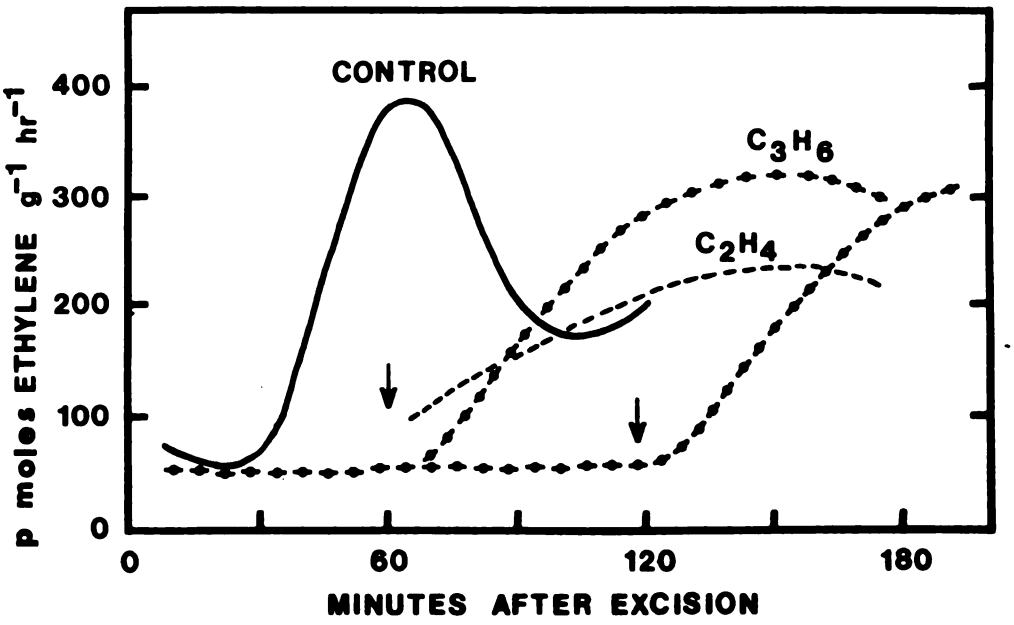

Figure 12. Effect of 11 ul $liter^{-1}$ ethylene or 500 ul $liter^{-1}$ propylene in air on wound-induced ethylene synthesis by subapical stem sections of etiolated 'Alaska' pea seedlings. The tissue was held under the appropriate gas mixture until the time designated by the arrows (60 min for both ethylene and propylene, and 120 min for propylene).

ethylene and propylene inhibit wound-induced ethylene synthesis, but do not inhibit induction of the wound response. If propylene was a competitive inhibitor of ethylene like CO_2, then the rate,but not the timing of the response, should have been changed. Yet the timing was actually more perturbed than the maximum rate of ethylene synthesis. These data indicate that propylene is an analogue of ethylene in this system, and that endogenous ethylene exerts negative feedback control on the rate of wound-induced ethylene synthesis. The second peak of wound ethylene can be explained on the basis of this idea.

The second peak of wound-induced ethylene synthesis may result from oscillations within the system as it recovers from the initial trauma. High levels of endogenous ethylene, resulting from the rise in production

during the first maximum, may cause deactivation of part of the system which results in decreased rates of production. As the internal concentration falls, more production may be activated and the rate of production again rises. The decline following the second maximum may not be the result of further negative feedback, but the result of depletion of a limiting substrate. This is supported by the observation that tissue in which the initial wound response had dissipated, and which were producing ethylene at a stable basal rate, were not stimulated to produce a second wound response when re-wounded. Apical sections appear to contain more of this substrate since they produce higher rates of wound ethylene and oscillate through more cycles (see section one). If the characteristic pattern is the result of negative feedback control, then it is obvious that a period of anaerobiosis before induction of wound ethylene would not perturb the pattern, while a similar period after induction would reduce the internal concentration of ethylene, modify the feedback control, and cause the observed radical perturbation of the response.

The reported surge in ethylene production following removal of tissue from anaerobiosis (15,20) may not be the result of the proposed accumulation of substrate, but rather the result of lowering the internal concentration of ethylene. Since anaerobic tissue does not produce ethylene, its internal concentration will equilibrate with the ambient atmosphere. The overshoot above the equilibrium rate of synthesis could therefore result from the slow response-time of negative feedback control on the rate of ethylene synthesis. Examination of the kinetics of wound-induced ethylene synthesis supports this hypothesis. The time between the inflection points of wound ethylene synthesis is around

35 min (start of increase to first peak = 30 min, first peak to minimum = 34 min, minimum to second peak = 41 min). The mid-point of each of these times, i.e. 15, 17, and 20 min before or after each inflection point, occurred at a production rate of about 290 p moles g^{-1} hr^{-1}. If the internal concentration is directly related to the rate of production, then it seems that the concentration associated with a production rate of 290 p moles g^{-1} hr^{-1} is the threshold for modulating ethylene synthesis, and that around 17 min is required to turn the synthesizing system on or off. A similar time lag of 17 min occurred in the surge of ethylene from aged subapical sections removed from anaerobiosis (Fig. 11). It is reasonable to conclude that the surge in ethylene synthesis following anaerobiosis is the result of reduced internal concentrations of ethylene and thereby reduced negative feedback control on the rate of ethylene synthesis, rather than the result of accumulated precursors.

Temperature Dependency. Temperature had a pronounced effect on the rate and timing of wound-induced ethylene synthesis (Fig. 13). The rate of production during the lag period was similar at temperatures of 10 C and 40 C. Between these temperatures, the rate of ethylene synthesis increased from 50 p moles g^{-1} hr^{-1} at 10 C to a maximum of 120 p moles g^{-1} hr^{-1} at 27 C to 30 C, and then declined to 60 p moles g^{-1} hr^{-1} at 40 C. The length of the lag period decreased from over 120 min at 10 C to a relatively constant 25 min at 30 C to 36 C. Above 36 C wound ethylene was completely inhibited, and only the basal rate was observed. The time to the first maximum decreased from over 120 min at 15 C to around 45 min at 30 C and then increased slightly to 50 min at 36 C. The rate of maximum wound ethylene synthesis increased from around

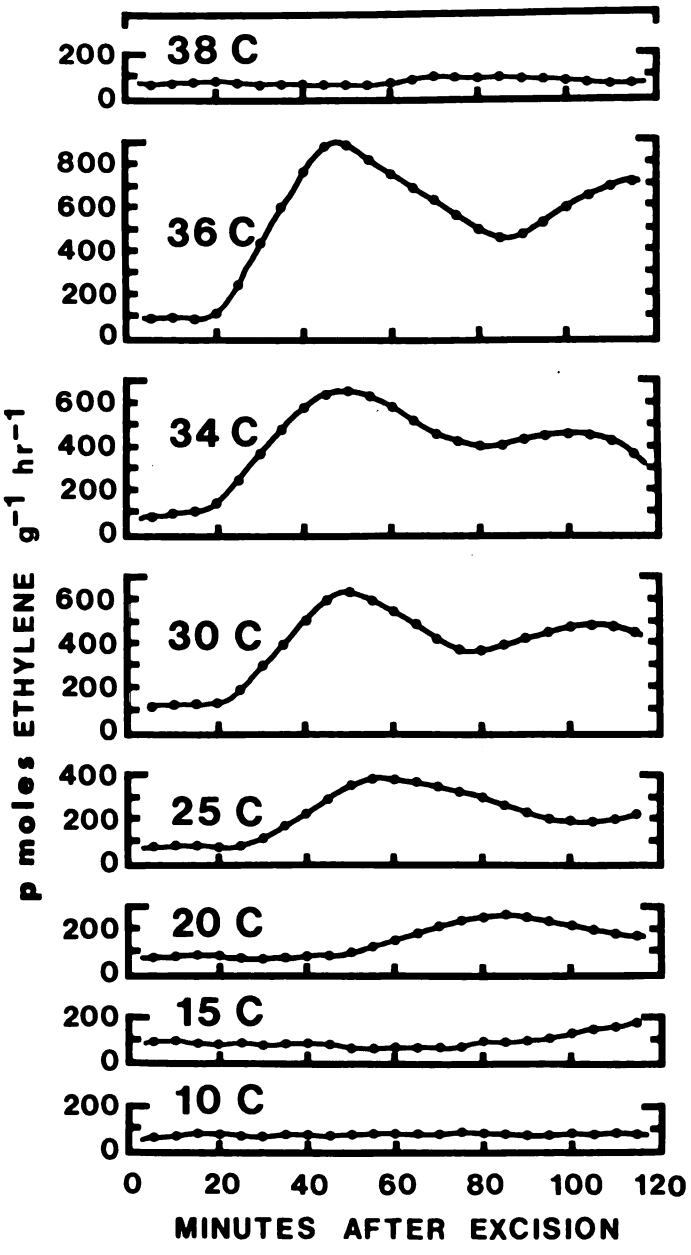

Figure 13. Effect of temperature on wound-induced ethylene synthesis by subapical stem sections of etiolated 'Alaska' pea seedlings.

40 p moles g^{-1} hr^{-1} at 10 C to 880 p moles g^{-1} hr^{-1} at 36 C.

An Arrhenius plot of the maximum rate of wound ethylene synthesis showed that an abrupt change from linearity occurred at 15 C and at 36 C (Fig. 14). A linear equation fitted to the points between 15 and 36 C

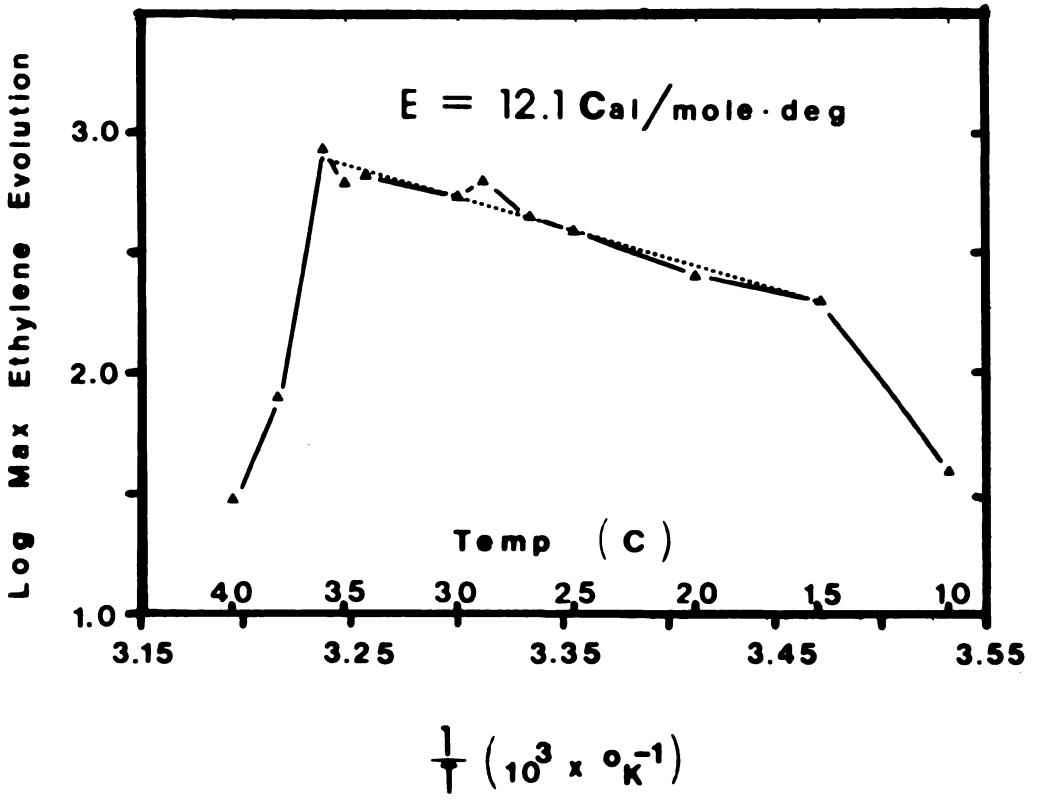

Figure 14. An Arrhenius plot of the rate of maximum wound-induced ethylene synthesis by subapical stem sections of etiolated 'Alaska' pea seedlings. A linear equation fitted to the points between 15 C and 36 C gave an activation of 12.1 Kcal mole^{-1} degree^{-1} for wound ethylene synthesis.

had an r^2 of 0.95 (N = 10). An activation energy of 12.1 Kcal mole^{-1} degree^{-1} was calculated from this equation. This activation energy corresponds to a temperature coefficient (a Q_{10}) of approximately 2.0 for temperatures between 15 and 36 C.

Although a change in the activation energy for respiration by peas does not occur between 9 and 12 C (35,48), a phase change in the components of the ethylene forming system in this temperature range could account for the observed deviation in activation energy. However, the phase change was not drastic since changing the temperature to 30 C,

after 100 min at 10 C, immediately initiated the characteristic pattern of wound ethylene synthesis. It appears that 10 C allows induction of the wound response, and drastically slows down the reactions necessary for wound ethylene synthesis.

Temperatures above 36 C reduce wound ethylene synthesis by more drastic means, such as denaturing or disrupting critical parts of the ethylene synthesizing system. This was shown by the observation that about 2 hr at 30 C was required for recovery from a 40 min exposure to 40 C. Exposure to 40 C for 1 min prolonged the lag period by 7 min, from 26 to 33 min. Exposure to 40 C for 5 min shifted the time of maximum wound ethylene production from 50 min to 80 min, broadened and reduced the maximum rate from 300 p moles g^{-1} hr^{-1} to 140 p moles g^{-1} hr^{-1}. Tissue held above 37.0 C did not produce any wound-induced ethylene. However, if the subapical sections were held at 36.0 C until the wound response was induced (i.e. for the 26 min lag period), then the temperature had to exceed 38.0 C to reduce wound ethylene synthesis. If the wound ethylene synthesizing system was associated with membranes, comparison of the phase change temperature of specific membrane fractions (e.g. plasmalemma, endoplasmic reticulum, tonoplast, mitochondria) could assist in localization of the site of wound ethylene synthesis.

Induction of Wound Ethylene Synthesis. It was observed in many experiments that subapical sections excised from stems previously perturbed by shaking, flexing, or cutting had slightly different kinetics of wound ethylene production than sections excised from undisturbed seedlings. The maximum rate of wound ethylene synthesis occurred 56 min after excision of subapical sections from freshly harvested 13 cm or 27 mm apical stem segments, or from 13 cm apical stem segments held in

a humid, ethylene-free atmosphere for 15 min. The maximum rate occurred at 51 min for subapical sections excised from 27 mm apical stem segments held for 15 min. The rate of maximum wound ethylene synthesis was slightly higher for subapical sections excised from freshly harvested tissue or from 13 cm apical segments held for 15 min (360 p moles g^{-1} hr^{-1}), than from 27 mm apical segments held for 15 min (330 p moles g^{-1} hr^{-1}), but the accumulation during the interval $i \pm 30$ min was not significantly affected by any of the treatments. This indicates that the cut used to prepare the 27 mm apical segment induced wound ethylene synthesis in the subapical region 9 mm away. If it had not, the maximum rate of wound ethylene synthesis wound have occurred at 71 min (56 min + 15 min) rather than the 51 min observed.

Cutting subapical stem sections in apical and basal halves showed that polarity of wound ethylene synthesis developed in the sections with time. Accumulation studies showed no significant difference among wound ethylene production by whole sections (3.7 nl g^{-1} hr^{-1}), sections cut in half (3.6 nl g^{-1} hr^{-1}), or the apical (4.2 nl g^{-1} hr^{-1}) or basal (3.8 nl g^{-1} hr^{-1}) halves during the 30 min following excision (the lag period). Differences became obvious during the 30 to 90 min collection interval, when the basal halves produced 51% more wound ethylene (10.3 nl g^{-1} hr^{-1}) than the apical halves (6.8 nl g^{-1} hr^{-1}). Ethylene production was the same for apical (6.9 nl g^{-1} hr^{-1}) and basal (7.2 nl g^{-1} hr^{-1}) halves during the 60 to 120 min collection interval. In the 90 to 150 min interval, the relative rates were reversed so that the apical halves produced 49% more ethylene (7.9 nl g^{-1} hr^{-1}) than the basal halves (5.3 nl g^{-1} hr^{-1}). Since sections excised nearer to the apex produce more wound ethylene and maintain higher rates of production for longer

periods of time (see section one), the observed difference in the interval of the second peak (90 to 150 min) can easily be explained. Similarly, apical halves should have produced more wound ethylene during the interval of the first peak (30 to 90 min). The higher rates of production by the basal halves could indicate that a factor necessary for wound ethylene synthesis accumulated in the basal half, or that cutting the section into smaller pieces differentially effected ethylene production in the apical and basal regions.

Removal of apical and basal 3 mm stem sections from 16 mm subapical sections at zero time or at 15 min after the initial excision used to prepare the 16 mm section, did not significantly effect the time or rate of maximum wound ethylene synthesis, or the accumulation of wound ethylene in the interval i $\pm$ 30 min (Table 4). This shows that accumulation

Table 4. Effect on wound ethylene synthesis of removing the apical and basal 3 mm from 16 mm subapical stem sections from etiolated 'Alaska' pea seedlings. The 16 mm section was excised 5 mm from the top of the apical hook. The 3 mm ends were removed at zero time or at 15 min after excision of the 16 mm section, and produced a 10 mm section of tissue from the same region of the pea stem as the normal 9 mm subapical section.

Time of Cut (min)		Wound-Induced Ethylene Synthesis[z]		
Apical	Basal	Time of Maximum (min)	Rate at Maximum (nl g^{-1} hr^{-1})	Accumulation (nl) (i $\pm$ 30 min)
0	0	57.6 $\pm$ 0.8	15.1 $\pm$ 2.3	5.2 $\pm$ 0.67
0	15	57.7 $\pm$ 1.9	14.1 $\pm$ 1.9	5.1 $\pm$ 0.53
15	0	58.2 $\pm$ 0.2	13.8 $\pm$ 1.8	5.0 $\pm$ 0.50
15	15	59.5 $\pm$ 1.3	12.4 $\pm$ 3.3	4.9 $\pm$ 0.79

[z] Quadratic equations were fitted to the data by the method of least squares, and used to calculate the rate and time of maximum ethylene synthesis, and the accumulation of ethylene in the interval i $\pm$ 30 min.

of a transportable substance at the apical or basal cut surfaces, did not account for the induction of, or significantly contribute to wound ethylene synthesis. Data in table 4 also shows that the wound stimulus was rapidly transmitted. If the excision cuts used to prepare the 16 mm subapical section were not sensed throughout the entire section, then the time of maximum wound ethylene production for sections trimmed at zero time would have occurred at 56 min, while those trimmed at 15 min would have occurred at 71 min (56 min + 15 min). However, the times of maximum wound ethylene production differ by only about 1 min, showing that the induction was from the initial cut.

Cutting may produce two zones of injured cells. Cells in zone one would be adjacent to the killed cells at the surface and would have a reduced rate of wound-induced ethylene synthesis. Cells in zone two would be farther away from the site of injury and would contain induced cells which would produce more ethylene per cell than in zone one. Cutting 9-mm subapical sections into smaller pieces was able to resolve the dimensions of these zones. Since subapical sections are not uniform in their potential to produce wound ethylene (differing from apical to basal regions), the sections were cut into equal parts to eliminate this confounding factor. Wound ethylene from whole sections or sections cut in half was about 0.043 nl mm^{-1} hr^{-1}. Cutting sections into thirds, fourths, or sixths reduced the rate of wound ethylene synthesis by about 30% to 0.029 $\pm$ 0.001 nl mm^{-1} hr^{-1}. These data imply that zone one must extend at least 1.5 mm, but not more than 2.25 mm, from the cut surface. They also imply that excision of the subapical section induces wound ethylene throughout the entire length section. If this were incorrect, additional wounding would increase wound ethylene

synthesis by inducing more cells; yet additional wounding decreased the rate of wound-induced ethylene synthesis.

The wound response is even more complex. Cutting subapical sections into smaller segments or removal of strips of epidermis not only decreased the maximum rate of wound ethylene production, but also delayed the onset of the response. The normal lag of 26 min was increased to 30 min for peeled subapical sections, and to 36 min for subapical sections cut into 2 mm sections. Both treatments reduced maximum rates of ethylene production by about 30%. The increased lag period may imply that it is a phase during which perturbed cells recover from the initial affect of the trauma. If zone one cells require longer to recover, then injury sufficient to produce all zone one cells would appear to delay the onset of wound-induced ethylene synthesis.

CONCLUSION

Similarities in the O_2 and temperature dependency of wound-induced ethylene synthesis by 9-mm subapical sections of etiolated 'Alaska' pea seedlings and ethylene synthesis by other systems, suggests that ethylene is formed by the same enzymatic system in both cases. However, other data suggest that wound ethylene synthesis is either produced in a different compartment of the cell, or is controlled by a different regulator. Wound ethylene synthesis had a higher half-maximal O_2 saturation (3.6%) than reported for ethylene synthesis by other tissues (around 2.0%). While O_2 is necessary for all physiologically produced ethylene, wound ethylene synthesis required O_2 for its induction during the lag period. Propylene, an active ethylene analogue, prevented

wound ethylene synthesis, but did not affect the basal rate of ethylene synthesis. Experiments showed that the rate of wound ethylene synthesis was controlled by the endogenous concentration of ethylene through negative feedback control. Temperature had a profound effect on the kinetics of wound ethylene synthesis, but had much less of an effect on the almost constant basal rate of ethylene production during the lag period. Since free-radical catalyzed reactions are relatively temperature independent, basal ethylene synthesis may be the result of an O_2 dependent free-radical system at a rate limiting step.

Induction of wound ethylene synthesis was by a rapidly transmitted signal since ethylene synthesis was induced throughout the entire subapical region by an excision cut made 2 cm away. Accumulation of a substance at the cut surfaces did not initiate wound ethylene, nor was the cut surfaces the site of enhanced synthesis. In fact, cells closer than about 2 mm to the cut surface produced about 30% less ethylene than induced cells farther than 2 mm from the cut surface. The speed at which the signal was transmitted suggests a physical or nerve-like electrical stimulus. Breaking the tension of the transporation system could be the signal, but little tension would be present in a 15 cm tall etiolated pea seedling at high humidity. Wounding has been shown to cause a rapid hyperpolarization of cells within about 30 min (26,36).

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SECTION THREE

STUDIES OF RAPIDLY INDUCED WOUND ETHYLENE SYNTHESIS BY EXCISED SECTIONS OF ETIOLATED *PISUM SATIVUM* L., CV. ALASKA

III. EFFECT OF IAA AND THE REQUIREMENT FOR AN UNIDENTIFIED DIFFUSIBLE FACTOR

EFFECT OF IAA AND
THE REQUIREMENT FOR AN UNIDENTIFIED DIFFUSIBLE FACTOR

ABSTRACT

Earlier studies (see sections one and two) established that excision of subapical stem sections from etiolated seedlings of Pisum sativum L., cv. Alaska, was sufficient injury to elicit a characteristic pattern of ethylene synthesis. At 25 C, wound-induced ethylene synthesis started to increase after a 26 min lag period, rose to a 4-fold maximum at 56 min, and then declined to a minimum at 90 min before rising to a lower second maximum at 131 min. Anaerobiosis stopped wound ethylene production and delayed the characteristic pattern by the length of time freshly excised tissue was deprived of O_2.

Applying chemicals which are known to perturb IAA transport, or applying IAA at low concentrations had no effect on wound ethylene synthesis. A water-soluble, heat-stable factor diffused from sections in contact with water or agar, which lowered the rate of wound ethylene synthesis, but did not effect the characteristic timing of the wound response. A bioassay was developed to study this diffusible factor. Precursors of ethylene synthesis in other tissue and in model systems (e.g. methionine, homoserine, homocysteine, propanal) did not prevent the loss of the capacity of subapical sections to produce wound ethylene in the bioassay. A few chemicals maintained normal rates of wound ethylene synthesis in sections in the bioassay (i.e. 10 uM kinetin or benzyladenine, 10 mM Ca,

23 nM triacontanol, and 200 mM sucrose). However, kinetin only was effective if supplied from the beginning of the anaerobic soak in the bioassay; kinetin had no effect if given after the sections had soaked in water for 30 min. Attempts to isolate the factor by solvent partition, dialysis, or molecular sieve or ion exchange chromatography of tissue extracts were unsuccessful.

INTRODUCTION

In two previous papers (see sections one and two) it was shown that excised 9-mm subapical sections from 7-day-old etiolated Pisum sativum L., cv Alaska seedlings go through a 2-cycle pattern of wound-induced ethylene synthesis in 4 hr. The rate of wound ethylene synthesis at 25 C started to increase after 26 min lag period, rose to a 4-fold maximum at 56 min, and declined to a minimum at 90 min before rising to a lower second maximum at 131 min. The first peak of wound ethylene synthesis (i.e. from 26 to 90 min) was not unique to 'Alaska' peas, but occurred at about the same time in subapical sections from a variety of etiolated seedlings. Anaerobiosis stopped wound ethylene synthesis and delayed the characteristic pattern by the length of time freshly excised tissue was deprived of O_2. No wound ethylene was synthesized at temperatures above 36 C or at 10 C. Between these temperatures the lag period and the time to maximum ethylene synthesis decreaseed, while the maximum rate of synthesis increased. Both the apical region of 13-cm apical stem segments aged for 18 hr, and 9-mm subapical sections which had once responded to wounding, did not produce a second wound response when cut. An analysis of data suggested that a factor which affects the rate, but

not the timing of wound ethylene synthesis was dissipated in these tissues.

Ethylene production by vegetative tissue has been reported to be dependent on the endogenous concentration of free auxin (1,5,8,11). Kinetin, which slightly increased ethylene production by itself, synergistically stimulated IAA-induced ethylene synthesis many-fold (9) by enhancing uptake of IAA and decreasing IAA conjugation (12). In the cyclic ethylene biosynthesis system proposed by Baur and Yang (3), homoserine and serine are the two necessary imputs needed to maintain ethylene production. Both are converted to methionine, which is then converted to ethylene. However, this may not be the pathway for synthesis of wound ethylene in pea seedlings. Burg and Clagett (6) have shown that although ethylene production by banana slices (Musa spp.) was stimulated about equally by L-methionine, DL-homoserine, and DL-homocysteine, etiolated pea seedlings only converted methionine to ethylene if they had been treated with IAA. Lieberman and Kunishi (14) described a system in which lipoxidase peroxidizes lipase-freed linolenic acid to propanal, which was then converted to ethylene and ethane by a copper containing enzyme. Feeding experiments (2,13,15) and the lack of the necessary constant ratio (14) between ethylene and ethane production (see section one) have indicated that this system is not functional in living cells.

This paper describes the effect of IAA and perturbing IAA transport on wound ethylene synthesis, and the attempted isolation and characterization of a diffusible factor necessary for wound-induced ethylene synthesis.

MATERIALS AND METHODS

Plant Material. Seven-day-old etiolated seedlings of 'Alaska' pea were grown and prepared as previously described (see section one). Apical stem sections were cut 9 mm, 3 cm, or 13 cm below the top of the apical hook, and included the apical meristem, plumule, apical hook, and a portion of the stem. All kinetic studies of wound-induced ethylene synthesis employed 9-mm subapical sections excised 9 mm below the top of the apical hook. Apical root sections were excised 2 cm from the apex of 5-day-old etiolated plants grown on moist paper toweling. A dynamic flow-through-system, or a static-accumulating-system were used to study the effects of various factors on the kinetics of wound ethylene synthesis. The procedures for identifying and quantifying ethylene, CO_2, and O_2 were the same as previously described (see section one).

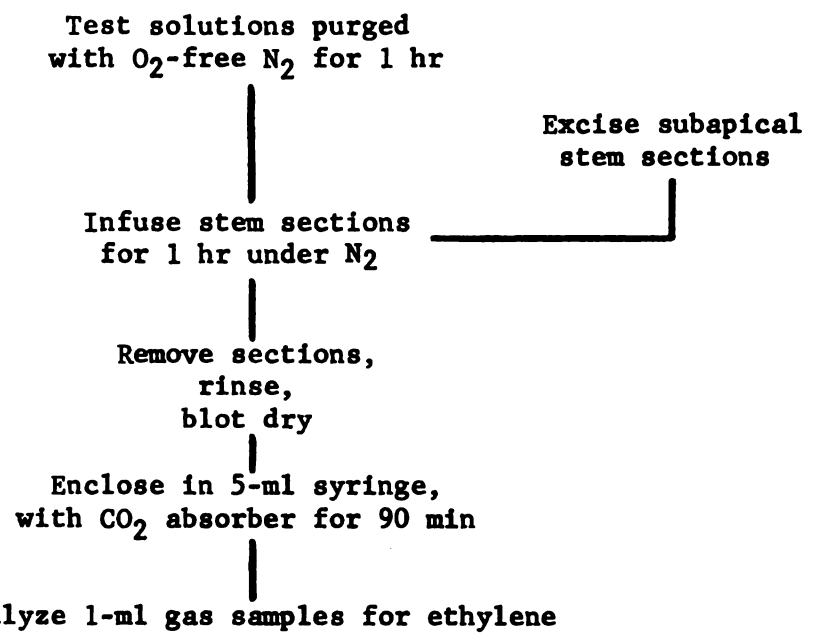
Perturbing Auxin Transport. The effect of auxin transport on wound ethylene production was investigated by applying inhibitors of auxin transport. Whole seedlings or 13-cm apical stem sections were dusted with an 80% wettable powder of DPX1840 (3,3 a-dihydro-2-(p-methoxyphenyl)-8H-pyrazolo(5,1-a)isoindol-8-one)(4), or 13-cm apical stem sections were dipped in 10 mM potassium phosphate buffer (pH 8.2) plus or minus 0.68 mM TIBA (triiodobenzoic acid) for 1 hr before excision of the subapical sections. Apical 13-cm stem sections were also allowed to take up buffer plus or minus 0.1 uM to 0.1 mM TIBA for 18 hr before excision of the subapical sections. Freshly excised subapical sections were dipped in buffer plus or minus 0.68 mM TIBA for 1 to 5 min, blotted dry, and the kinetics of wound ethylene synthesis was studied. Agar blocks (0.25%, w/v) plus or minus 0.50 mM TIBA were applied to the apical and/or

basal ends of freshly excised subapical sections which were positioned vertically in glass holders. Silicon stopcock grease was applied to the cut ends not covered with agar to eliminate differences in gas diffusion from the tissue. The kinetics of wound ethylene synthesis were monitored for 90 min after excision or after treatment.

Effect of Applied Auxin. Subapical sections were positioned vertically in glass holders and dipped for 5 min in 10 mM potassium phosphate buffer (pH 6.8) plus or minus 1.0 uM IAA. All free water was blotted away from the sections and the kinetics of wound ethylene synthesis were followed for 90 min. The effect of IAA on wound ethylene synthesis was also studied using a bioassay.

Bioassay. The bioassay procedure is outlined in Figure 15. About 33 subapical sections were put into 125-ml Erlenmeyer flasks containing

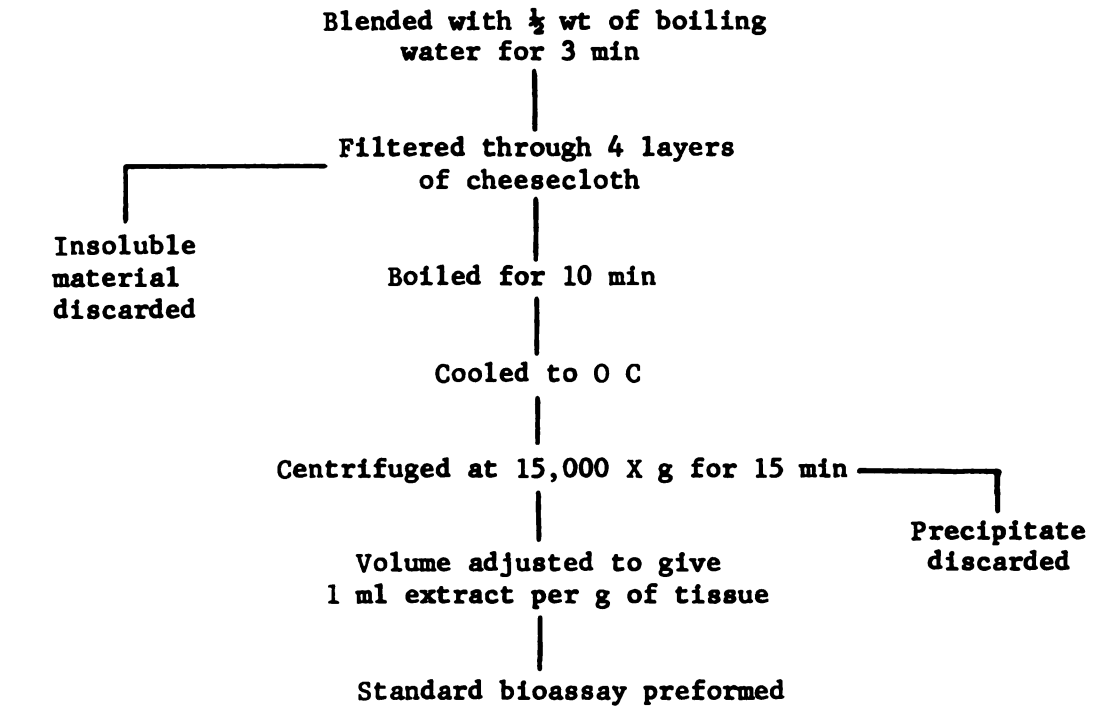
Figure 15. Flow chart of the bioassay used to test various solutions for their ability to prevent reduction of wound ethylene synthesis by subapical sections in contact with water.



50 ml of test solution (Tables 6,7,8,9). The control flasks contained moist filter paper. All flasks were purged with ethylene- and O_2-free N_2 for 1 hr before freshly excised subapical sections were added. The flasks were then maintained anaerobic for 1 hr with a reduced flow of N_2. The sections were removed, rinsed with distilled water, blotted dry, and 10 sections were put in each of three 5-ml plastic gas-tight syringes. A 1 cm^2 piece of filter paper, moistened with freshly prepared saturated KOH, was included to absorb CO_2. The syringes were set to 5 ml and capped with rubbed serum stoppers. All previous operations were preformed in the dark or under dim green light. One-ml gas samples were taken after 90 min and analyzed for ethylene and CO_2. The syringes were taken apart and the tissue weighed. Wound ethylene synthesis was calculated as nl g^{-1} hr^{-1}.

Isolation of the Diffusible Factor. Initially, infusions were made by putting apical and/or subapical stem sections in distilled-deionized water purged with O_2-free N_2. Later, stem and root tissue extracts were made using the procedure shown in Figure 16. Either fresh or frozen tissue was blended for 3 min with one-half its weight of boiling water. Some extracts were made at 0 C, but produced a less active extract. After filtering the brei through four layers of cheesecloth, the clear filtrate was boiled for 10 min to reduce its volume. After cooling to 0 C (ca. 1 hr), the mixture was centrifuged at 15,000 X g for 15 min and the precipitate, which had low activity in the bioassay, was discarded. The clear, amber-colored liquid was either reduced in volume in vacuo at 60 C, or water was added to give a concentration of 1 ml extract per g of tissue. The resultant crude extract had a pH of 6.0 and about 25 mg dry weight per ml. A bioassay was preformed to assay the activity of the extract.

Figure 16. Flow chart for the preparation of crude extracts from etiolated apical stem or root tissue.



RESULTS AND DISCUSSION

Wound-Induced Ethylene Synthesis and Auxin. Since apical sections produce higher rates of wound ethylene for prolonged periods (see section one), and since auxin is synthesized in the apical region and rapidly decreases in concentration basipetally (16), IAA was proposed as an active factor in the synthesis of wound-induced ethylene. However, none of the experiments reported in this paper support this hypothesis. Inhibition of auxin transport with TIBA or DPX1840 failed to change the timing or rates of wound ethylene synthesis (Table 5). This is in agreement with

previous results that accumulation of a substrate at a cut surface was not necessary for induction of wound ethylene, nor that the cut surfaces produced the greatest amount of wound ethylene (see section two). It is true that removal of the apical 5 mm, and with it the source of IAA, will reduce the ability of subapical sections excised 4 hr later to make wound ethylene (from 8.0 to 5.8 nl g^{-1} hr^{-1}). However, excision of the apical 5 mm may have induced a wound response, so that subapical sections excised 4 hr later were unable to produce wound ethylene because of this prior induction of the wound response, rather than because of lowered IAA content. An excision cut 3 cm below the apical hook also caused a decrease in wound ethylene production by subapical sections excised 2 hr later (see section one). Application of IAA at levels below the threshold to induce ethylene production (1 uM), also failed to elicit a second wound response or in any way alter wound ethylene production. During these experiments it was observed that sections produced much less wound ethylene if their cut ends were in contact with water or 0.25% agar (Table 5).

The diffusible factor. Since the kinetics of wound-induced ethylene synthesis are delayed by the length of time freshly cut sections are deprived of O_2 (see section two), a bioassay employing an anaerobic soak was used to study the effect, on the kinetics of wound ethylene production, of soaking excised tissue segments in various solutions (Tables 6,7,8,9). Since none of the soaking solutions significantly effected the characteristic timing of the response, wound ethylene production was studied with 90 min accumulation experiments. The rate of wound ethylene production was reduced by 75%, for subapical sections held for 1 hr in a humid N_2 atmosphere (3.8 nl g^{-1} hr^{-1}) as compared to

Table 5. Effect on wound-induced ethylene synthesis of applying silicon stopcock grease or 0.25% agar plus or minus 0.68 mM TIBA to the apical or basal cut ends of subapical sections of etiolated 'Alaska' pea seedlings.

Treatment of Cut Surface	nl Ethylene g^{-1} hr^{-1}	
Apical / Basal	No TIBA	0.68 mM TIBA
Silicon grease / Silicon grease	5.03 a[z]	----
Silicon grease / 0.25% Agar	3.74 b	4.01 b
0.25% Agar / Silicon grease	3.70 b	3.63 b
0.25% Agar / 0.25% Agar	3.04 c	2.62 c

[z] Means in the same row or column followed by the same letter are not significantly different at the 0.05 level.

sections held for 1 hr in 150 ml of N_2 purged water (0.9 nl g^{-1} hr^{-1}). Apical 9-mm stem sections showed only a 35% reduction in wound ethylene production under similar conditions. Burg and Thimann (7) showed that ethylene production from apple (Malus domestica Borkh.) tissue was extremely sensitive to osmotic shock. They found that while slight dehydration had no effect, hypotonic solutions destroyed the ability of apple tissue to produce ethylene. This was not the cause of reduced wound ethylene synthesis in subapical sections since placing agar on the cut ends also diminished synthesis (Table 5). Placing agar on the apical and basal cut ends of subapical sections reduced wound ethylene by 40%, while placing agar on either the apical or the basal ends only reduced production by 20%. This reduction was not the result of blocked

diffusion since silicon stopcock grease applied to the uncovered end(s) did not significantly affect wound ethylene synthesis as compared to uncovered controls.

Various chemicals were tested using the bioassay to see if they would replace or prevent the loss of wound ethylene production capacity (Table 6). Precursors of ethylene synthesis in other tissue and in model systems did not prevent loss of wound ethylene production. Concentrated Hoagland nutrient solution (10) had no significant effect, which indicates that the factor was not an inorganic nutrient. Apart from 100 uM IAA, which is above the threshold for induction of ethylene production, and 100 mM propanal, which is a non-physiological concentration, only those chemicals which assist in maintaining membrane integrity (i.e. kinetin, benzyladenine, calcium, triacontanol, and high levels of sucrose and galactose) prevented reduction in wound ethylene production to the level of the water soaked control. Feeding 13-cm apical stem segments with 10 or 100 mM sucrose significantly increased the rate of wound ethylene synthesis, from 0.8 ± 0.2 nl g^{-1} hr^{-1} to 4.1 ± 0.3 nl g^{-1} hr^{-1}, by subapical sections excised from the stems 18 hr later. Subapical sections anaerobically soaked in 10 mM kinetin or benzyladenine did not show any loss of wound ethylene production capacity over the moist controls (Table 7), and had similar kinetics of synthesis when returned to air (Fig. 17).

Apical and subapical sections were placed in dialysis bags with O_2-free water and kept in an equal volume of water under O_2-free N_2 for 2.5 hr. A bioassay was run on 50 ml aliquots of the diffusate and of the solution in the dialysis bags. The diffusate reduced wound ethylene synthesis to the same degree as the water soaked control, while the

Table 6. Effect of various chemicals on wound-induced ethylene production. Subapical sections were excised, held for 1 hr in solutions flushed with nitrogen, transfered to 5-ml syringes, and ethylene assayed after 90 min accumulation in air. Results are given as a percentage of the non-soaked control.

Treatment Solution	Ethylene Production (as % control)
Moist control	100 b[z]
Water soaked	25 d
Hormones (uM)	
IAA (1.0)	28 d
IAA (100)	450 a
Benzyladenine (10)	93 b
Benzyladenine (100)	116 b
Kinetin (10)	94 b
GA_3 (10)	23 d
GA_3 (100)	20 d
GA_3 (100) + BA (10)	108 b
Amino acids (mM)	
Methionine (1.0)	27 d
Methionine (50)	23 d
Homoserine (1.0)	28 d
Homoserine (20)	26 d
Homocysteine (1.0)	30 d
Sugars (mM)	
Galactose (1.0)	34 d
Galactose (100)	47 c
Sucrose (1.0)	31 d
Sucrose (100)	51 c
Sucrose (200) + $CaCl_3$ (10)	101 b
Other compounds	
Propanal (1.0 mM)	21 d
Propanal (10 mM)	25 d
Propanal (100 mM)	44 c
Triacontanol (23 nM)	86 b
Hoagland nutrient solution (5X)	23 d
Hoagland micronutrients (10X)	27 d

[z] Means followed by the same letter are not significantly different at the 0.05 level.

Table 7. Effect on wound-induced ethylene synthesis in the bioassay of various concentrations of benzyladenine.

Treatment (uM benzyladenine)	Wound ethylene synthesis (nl g^{-1} hr^{-1})
Water soaked control	1.79 ab [z]
0.01	1.62 a
0.1	2.05 b
1.0	2.26 b
10.0	2.61 c
100.0	2.77 c
Moist control	2.64 c

[z] Means followed by the same letter are not significantly different at the 0.05 level.

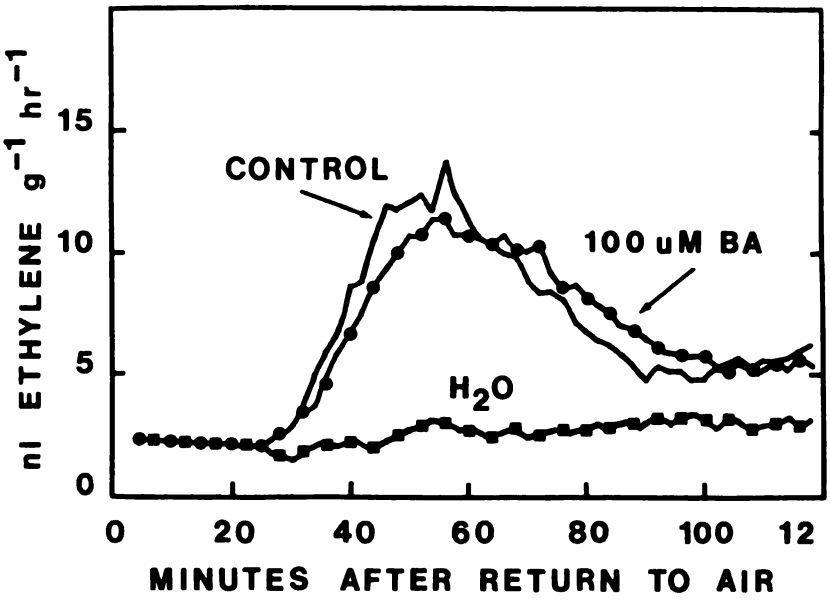


Figure 17. Effect of holding subapical stem sections anaerobically for 1 hr in a humid atmosphere (control) or in water (H_2O) plus or minus 100 uM benzyladenine (BA), on the kinetics of wound-induced ethylene synthesis when the sections were returned to air.

solution in the dialysis bag inhibited wound ethylene synthesis an additional 30% below the water soaked control.

If reduction of wound ethylene synthesis resulted from the diffusion of a substance out of the tissue, then placing sections in a solution could prevent the reduction by either preventing the loss of the substance, or by replacing, by back diffusion, the substance that was lost. The mechanism which prevented the reduction in wound ethylene synthesis was resolved by applying test substances 30 min after the beginning of the anaerobic soak. If wound ethylene production capacity was reinstated, it would indicate that the solution contained the substance which had diffused out of the tissue, in sufficient concentration to maintain an adequate concentration of the substance within the tissue.

Using this criterion, it was shown that cytokinins prevented the loss of some factor from induced cells, but were not the factor. Application of 10 uM kinetin to sections after they had soaked anaerobically in water for 30 min failed to maintain wound ethylene production.

Attempts to isolate the diffusible substance from anaerobic infusions or from tissue extracts were only partially successful. Infusions of apical and subapical stem sections showed that the substance was stable to boiling, and was more concentrated in the apical than in the subapical sections. Since apical sections also produce more wound ethylene, these bioassay data support the contention that a substance necessary for wound ethylene production was being isolated. However, this method did not provide a sufficient volume of liquid with enough activity to permit testing partitioned fractions with the bioassay. To provide enough material, aqueous tissue extracts were prepared. The extraction procedure (Fig. 16) produced a clear, amber-colored liquid

at pH 6.0 and with about 25 mg dry weight per ml. Extracts prepared at 0 C were less active. Extracts of roots, and to a lesser extent stems, had the greatest effectiveness in maintaining wound ethylene production capacity in the bioassay (Table 8). Subapical sections kept in the root

Table 8. Relative effectiveness of various tissue extracts on the retention of wound-induced ethylene production capacity in the bioassay.

Treatment	Ethylene production (as % control)
Freshly cut sections	217
Extract from root apices	214
Extract from stem apices	120
Moist control	100
Extract from 3 cm apical stems	86
Extract from 2nd internode tissue	41

extract for 2 hr under N_2 had, when moved to air, kinetics of wound ethylene production similar to sections freshly excised, held in a moist anaerobic atmosphere for 2 hr, or held anaerobically in 100 uM benzyladenine for 2 hr (Fig. 17). Since extracts from 3 cm apical stem segments were more easily prepared, they were used in most of the fractionation studies.

Since the cytokinins,kinetin and benzyladenine were some of the most effective chemicals tested (Table 6), and since pea roots, which had the highest extractable activity (Table 8), are also high in cytokinins (17), the extract was treated to isolate cytokinins by the method

of Short and Torrey (17). However, the crude fraction contained so much material that their method did not work. Attempts to purify the active factor with ethanol or NH_4OH precipitation, either diluted or destroyed the activity without concentrating it in any one fraction (Table 9). Attempts to isolate the active factor by various other

Table 9. Effect of boiling and various fractionation procedures on the activity of fractions of 3-cm apical-stem-extracts on wound ethylene production using the bioassay. Results reported as percentage of crude extract activity in assayed fraction.

Treatment	Ethylene production (as % extract)
Extract from 3 cm apical stems	100
Boiled extract	96
Ethyl ether pH 10	60
Ethyl ether pH 7	49
Ethyl ether pH 3	64
Aqueous phase	66
Ethanol precipitate, 60%	39
Ethanol precipitate, 60% - 80%	54
Ethanol precipitate, 80% - 90%	53
Aqueous phase	29
Hexane	51
Ethyl acetate	40
Aqueous phase	57
Dialysis (interior)	28
Dialysis (exterior)	41
Cation exchange column (H^+)	
0 - 50 ml (loading)	30
50 - 100 ml (water wash)	69
100 - 150 ml (0.05 N HCl)	20
150 - 200 ml (0.05 N HCl)	34
200 - 250 ml (1.0 N HCl)	17

Table 9. (cont'd).

Treatment	Ethylene production (as % extract)	
Cation exchange column (Na^+)		
0 - 50 ml (loading)	45	
50 - 100 ml (water wash)	52	
100 - 150 ml (0.1 M KNO_3)	40	
Anion exchange column (OH^-)		
0 - 50 ml (loading)	51	
50 - 100 ml (water wash)	31	
100 - 150 ml (0.05 N NaOH)	48	
Anion exchange column (CH_3COO^-)		
0 - 50 ml (loading)	43	
50 - 100 ml (water wash)	50	
100 - 150 ml (0.1 M KNO_3)	36	
Molecular sieve chromatography (V_o = 50 ml)		O.D. 260 nm
50 - 60 ml	37	0.00
60 - 70 ml	25	0.00
70 - 80 ml	33	0.01
80 - 90 ml	34	0.02
90 - 100 ml	34	0.00
100 - 110 ml	27	0.00
110 - 120 ml	45	0.01
120 - 130 ml	33	0.28
130 - 140 ml	31	1.42
140 - 150 ml	49	2.85
150 - 160 ml	57	2.97
160 - 170 ml	42	1.31
170 - 180 ml	53	1.38
180 - 190 ml	43	0.48
190 - 200 ml	47	0.47
200 - 210 ml	43	0.48
210 - 220 ml	37	0.52
220 - 230 ml	48	0.61
230 - 240 ml	50	0.60

fractionation methods (ether partitioning at pH 3, 7, 10, hexane and ethyl acetate partitioning, molecular sieve chromatography (Bio-Gel P-2), cation (Dowex 50W-X8) and anion (Dowex 1-X8) exchange chromatography) were also unsuccessful (Table 9).

CONCLUSION

A number of experiments, which were logical extensions of the present studies, were not performed because the wound response could not be maintained under the treatment conditions. Feeding labeled chemicals, or inhibitors of RNA, protein, or ethylene synthesis require soaking the tissue in solutions. Soaking by itself, has been shown to markedly reduce wound ethylene production. It is true that the response can be maintained with 10 uM benzyladenine or crude tissue extracts, but since the mechanism of action of BA was not determined and the extract's composition was unknown, experiments with either addition would have produced ambiguous results.

The wound response was unaffected by chemicals known to perturb IAA translocation and by applied IAA below the induction threshold for ethylene synthesis. Application of precursors of ethylene synthesis in other systems failed to maintain wound ethylene production in the bioassay. Applied cytokinin prevented the loss of wound ethylene production capacity in the bioassay, but only did so if applied from the beginning of the anaerobic soak. A heat-stable, water-soluble factor which could also prevent loss of wound ethylene production capacity was isolated in crude fractions, but attempts at purification were unsuccessful. The inductor(s) and precursor(s) of wound ethylene synthesis in etiolated peas remains unknown.

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SUMMARY AND CONCLUSIONS

Wounding or excising tissue from the apical region of etiolated 'Alaska' pea seedlings rapidly initiated a transitory increase in the rate of ethylene synthesis. In 9-mm subapical sections excised 9 mm from the top of the apical hook, the characteristic pattern at 25 C involved a 26 min lag period before increased synthesis produced a major peak at 56 min, a minimum at 90 min, and a minor second peak at 131 min. The wound signal which initiated wound ethylene synthesis, was quickly transmitted through the pea stem since the entire apical region was uniformaly induced to make wound ethylene by cuts made 2 or more cm away, in parts of the stem which did not produce measureable wound ethylene. Cells closer than 2 mm to the cut surface synthesized 50% less wound ethylene, than induced cells greater than 2 mm from the cut surface. As the temperature increased from 10 C to 36 C, the lag period and the time to the first maximum of wound ethylene production decreased to a constant time for temperatures between 30 C and 36 C, while the maximum rate of synthesis continued to increase up to 36 C. Temperatures above 36 C or below 15 C stopped wound ethylene synthesis, but did not prevent evolution of a low basal rate of ethylene.

Data indicated that the minimum in wound ethylene synthesis at 90 min resulted from negative feedback control by endogenous ethylene on the rate of wound-induced ethylene synthesis, while the decline to non-wounded levels of synthesis, after about 3 hr, resulted from depletion

of a necessary factor. Propylene delayed the rise in wound ethylene synthesis, but allowed induction of the wound response and did not affect the basal rate of synthesis. Aged tissue, tissue which had once been induced to produce wound ethylene, and tissue soaked anaerobically in water did not produce wound ethylene when wounded, re-wounded, or returned to air. However, they all produced a low basal rate of ethylene. It was proposed that the loss of the capacity to synthesize wound ethylene in those tissues resulted from the loss of a common factor. A factor was isolated in infusions of subapical pea stems, and in crude extracts of pea roots and stems which maintained wound ethylene production capacity in a bioassay. Application of precursors of ethylene synthesis in other tissue and in model systems failed to maintain wound ethylene production capacity in the bioassay. Applied cytokinins prevent the loss of wound ethylene production capacity in the bioassay, but were effective only if applied from the beginning of the anaerobic soak. Attempts to purify the factor were unsuccessful.

The continued presence of a basal rate of ethylene synthesis at 10 C and at 37 C, and under applied propylene suggests that there are two ethylene forming systems. One produces wound ethylene and resembles a physiological system since it is temperature sensitive with a Q_{10} of about 2.0, and requires O_2 for its induction and continued synthesis of ethylene. The other system resembles an O_2 dependent free-radical catalyzed reaction since it is relatively temperature independent, and requires no induction.

Wound-induced ethylene synthesis appears to be a part of the mechanism plants have evolved for dealing with injury. Since all parts of the plant have the metabolic capacity to respond to wounding, the initial

response must be localized to minimize disruption of normal metabolism. A gaseous hormone is ideally suited to mediate wound healing. No active control of transport or deactivation is necessary. Natural diffusion and dissipation of ethylene limits induction to the immediate area. What metabolic processes are induced by ethylene, and how these function in repair or defense of damaged tissue remain to be clarified.